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MYCOPARASITISM OF *ALTERNARIA BRASSICAE* BY *NECTRIA INVENTA*

by

© AKIHIKO TSUNEDA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled MYCOPARASITISM OF *ALTERNARIA BRASSICAE* BY *NECTRIA INVENTA* submitted by AKIHIKO TSUNEDA in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Pathology.

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ABSTRACT

More than 30 genera of fungi were isolated from the phylloplane of two cultivars of rapeseed, i.e., Torch (*Brassica campestris* L.) and Midas (*B. napus* L.). Among them *Alternaria alternata* (Fr.) Keissler, *Cladosporium herbarum* (Pers.) Link ex S.F. Gray, and *Fusarium avenaceum* (Corda ex Fr.) Sacc. were the dominant fungi, followed by the *Verticillium* state of *Nectria inventa* Pethybridge.

Among the major phylloplane fungi tested in culture, the conidial *N. inventa* was the most destructive parasite of *A. brassicae* (Berk.) Sacc., the causal fungus of blackspot of rapeseed. Other fungi which were antagonistic to *A. brassicae* were *Chaetomium olivaceum* Cooke & Ellis, *Rhizopus oryzae* Went & Geerligs, *Trichoderma harzianum* Rifai, and *Trichothecium roseum* Link.

In the *A. brassicae* - *N. inventa* host-parasite interaction, tropic growth of parasite hyphae towards host hyphae and conidia occurred in the vicinity of the host. Parasitism took place subsequently either by means of contact without penetration or by penetration of the host. Upon contact, the parasite hyphae often formed appressorium-like bodies on the host cells, produced fibrous adhesive material at the host-parasite interface, and induced abnormal responses in the host cell. A reaction zone that consisted largely of an electron-transparent matrix with dispersed tubule-like electron-dense material developed between the

host cell wall and the invaginated plasma membrane. The tubule-like elements subsequently aggregated to form electron-dense deposits below the cell wall. The affected cell formed a septal plug, accumulated membranes in some cases, and finally degenerated. Conidia were penetrated more often than hyphae. Penetration occurred commonly at the septa and appeared to be primarily chemical in nature. The cytoplasm of the invaded cell became progressively less dense and the cell eventually became empty.

Conidia of *A. brassicae* often formed microsclerotia or endocellular chlamydospores after cold temperature treatment or during gradual desiccation in culture. Mature microsclerotia were darkly pigmented, many-celled, unevenly spheroidal, and about 60-150 µm in diameter. Microsclerotia and endocellular chlamydospores were parasitized by *N. inventa*.

Parasitism of *A. brassicae* by *N. inventa* occurred most vigorously when the temperature was in the range of 20-28°C.

Conidia of *A. brassicae* leaked various amino acids, e.g., glutamine, aspartic acid and glutamic acid, and sugars, glucose and fructose, after they were exposed to an alternate dry-wet condition. These leaked nutrients stimulated growth of *N. inventa*, which then destroyed the host conidia.

On intact rapeseed leaves, *N. inventa* did not prevent the primary infection of the leaves by *A. brassicae*. Blackspot lesions developed within 48 hours after inoculation, but *N. inventa* required at least 4 days to initiate parasitism. In contrast, *N. inventa* suppressed the vegetative growth and sporulation of *A. brassicae* on excised rapeseed leaves.

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GENERAL INTRODUCTION

Rapeseed is now firmly established as Canada's third most valuable crop, following wheat and barley (Downey, Klassen, and McAnsh, 1974). Significant progress has been made in improving the agronomic characteristics of this crop. However, many cultivars commonly grown in the prairie provinces are still susceptible to a variety of pathogens.

Blackspot of rapeseed, caused by *Alternaria brassicae* (Berk.) Sacc. is one of the major diseases that causes substantial reduction in crop yield and in yield and quality of oil (Degenhardt, Skoropad, and Kondra, 1974). According to recent disease surveys in Alberta, however, the incidence of this disease has been shown to fluctuate considerably between different years. The reason for this fluctuation could not be explained readily on the basis of weather changes.

It was speculated, therefore, that not only some abiotic factors, such as weather conditions, but biotic agents associated with rapeseed could also contribute to this situation. The possibility that phylloplane mycoflora could be involved in some way in suppressing the pathogen was the principal motive of this study.

This paper consists of 2 main sections: (I) Isolation of phylloplane fungi from rapeseed, and examination of antagonistic abilities of major isolates to *A. brassicae*, and (II) A detailed study of the host-parasite interaction, i.e.,

mycoparasitism. *Nectria inventa* Pethybridge was found to be strongly parasitic to *A. brassicae* and, therefore, the mode of this interaction was investigated. Factors influencing their interaction and the possibility of effecting biological control of the pathogen by using *N. inventa* were also examined.

GENERAL LITERATURE REVIEW

Rapeseed Cultivars

The two species of rapeseed commonly grown in Canada are *Brassica napus* L. (Argentine-type) and *B. campestris* L. (Polish-type). In general, the Argentine types mature 2-3 weeks later than the Polish types. The growth-stage key of rapeseed is given by Berkenkamp (1973). Argentine rapeseed has a higher yield potential and is more resistant to most of the important diseases (Downey, Klassen, and McAnsh, 1974). The distribution of these two types on the Canadian prairies depends largely on the length of the growing season. In central and northern Alberta the Polish type cultivars are grown predominantly.

Alternaria Blackspot of Rapeseed

Two *Alternaria* species, i.e., *A. brassicae* and *A. raphani* Groves and Skolko, cause blackspot of rapeseed (Taber and Vanterpool, 1963). In Alberta, however, *A. raphani* seldom occurs on blackspot lesions (Personal communication with Dr. J.P. Tewari).

The pathogens causing blackspot attack all cultivars of *B. campestris* and *B. napus* grown in Canada (Downey, Klassen and McAnsh, 1974). Tewari and Skoropad (1976) reported, however, that cultivars of *B. napus*, Iowa line and Midas, are more resistant to *A. brassicae* than Torch (*B. campestris*). They concluded that epicuticular wax, richer in Iowa line and Midas than in Torch, confers partial resistance to the pathogen through a more effective water repellency.

The first visible symptom of this disease is brown or black dots on leaves and/or on stems. These develop into prominent spots or elongate lesions of various sizes, which may be entirely brown or black or grayish-white (Petrie, 1972). Leaf spots are often surrounded by chlorotic areas. The pathogens also infect pods, where they cause premature splitting and seed shrinkage (Petrie, 1972 and 1975). These effects result in a reduction in yield of rapeseed crop and a lowering of yield and quality of oil (Degenhardt, Skoropad, and Kondra, 1974). In Saskatchewan, for example, over 90% of the plants were spotted and yield losses of over 20% were reported during 1970 to 1972 (Petrie, 1973). Similarly in Manitoba in 1972, blackspot was found in 95% of the fields of Polish-type rapeseed and in 78% of the fields sown to the Argentine-type rapeseed (Platford and Bernier, 1973).

A critical historical review on the taxonomy and nomenclature of *Alternaria* species including the blackspot pathogens was given by Wiltshire (1947) and Simmons (1952),

and a detailed taxonomic description by Groves and Skolko (1944), Neergaard (1945), Joly (1964), and Ellis (1971). A comparative physiological study of *A. brassicae* and *A. raphani* has been done by Taber, Vanterpool, and Taber (1968).

Nectria inventa

Nectria inventa was first discovered by Pethybridge in 1919 in England. He found many dark red perithecia on the surface of some old seed potatoes. Ascospores were two-celled and a stroma was present. Based on these characteristics, the fungus was identified as *Nectria* and since the perithecia were discovered more or less by accident, the specific name "inventa" was adopted. After further studies of this fungus in pure culture, using single ascospores, he reported its conidial state as *Verticillium cinnabarinum* Reinke & Berth. Pethybridge (1919) therefore recommended that this species be removed from the Class, Fungi Imperfecti and be placed in the Class, Ascomycetes. However, the ascospores have not been reproduced in culture, and his work has not been verified (Booth, 1959).

There is no general agreement on the taxonomic disposition of the conidial state of *N. inventa*. Hughes (1951) examined the conidial apparatus of this fungus and suggested that conidial *N. inventa* is most probably the fungus upon which Nees (1817) based his species *V. tenerum* Nees ex Link, which is the type species of the genus *Verticillium*. In addition to *V. cinnabarinum* and

V. tenerum, the conidial state of *N. inventa* has been referred to as *Acrostalagmas cinnabarinus* Corda and *V. lateritium* Berk. Barron (1968) stated that the latter name is probably the best taxonomic disposition for conidial *N. inventa*. In this thesis, it was decided to follow the suggestion made by Pethybridge (1919) and therefore the name of the perfect stage is used for the fungus throughout.

The conidial state of *N. inventa* is a very common saprophyte (Isaac, 1967) and occurs on a wide variety of substrata. A detailed description of conidial *N. inventa* and a list of substrata are reported in a paper by Hughes (1951).

Microbial Relationships

In natural environments, a number of relationships exist between individual microbial species and between individual cells. The composition of microflora of any habitat is governed by the biological equilibrium created by the associations and interactions of all individuals found in the population. However, since microbial associations are usually highly complex and sensitive to slight changes in the environment, the equilibrium cannot be stable in nature. It is constantly being re-established in a modified form.

In the past a number of terms have been employed to designate relationships between organisms. The terminology, however, is somewhat confusing. In this thesis, the term "antagonism" is used to include three types of activity: (1) antibiosis

and lysis; (2) competition; and (3) parasitism and predation (Baker and Cook, 1974). Antibiosis is the inhibition of one organism by a metabolic product of another. Lysis is a general term for the destruction, disintegration, dissolution, or decomposition of biological materials (Lamanna and Malette, 1965). Competition implies a condition in which there is a suppression of one organism as the two species struggle for limiting quantities of nutrients, oxygen, space, or other common requirements. Parasitism and predation indicate the direct attack of one organism upon another. The term "parasite" is used if the organism actually lives in or on the host, which, therefore, is both the energy source and a habitat. In the case of predation, the prey serves as an energy source but not as habitat (Odum, 1963 and 1971).

Phylloplane Fungi and Phytopathogenic Alternaria

The presence of fungi on the surface of plants has been recognized for more than a century, and it is now common knowledge that leaf surfaces are covered with large numbers of different kinds of micro-organisms including spores, hyphae, yeast cells and bacteria (Dickinson, 1973).

As a habitat for micro-organisms, the surface of plant leaves has been commonly called "phyllosphere" (Last, 1955; Ruinen, 1963); however, Kerling (1958) suggested the term "phylloplane" instead, and this terminology is being used more widely. The plant pathogenic fungi must spend a

critical period of time on the phylloplane before they can cause infection. Similarly, saprophytes and weak parasites, which begin the decomposition of the tissue, must wait until the host's resistance is lowered, or the onset of senescence allows the initial penetration into the tissues (Pugh and Buckley, 1971).

Potter (1910) suggested that non-pathogenic phylloplane organisms may be important in determining the severity of plant diseases incited by fungi on leaves. Since then, the antagonistic effects of non-pathogenic microorganisms on plant pathogens have been reported by many investigators, and reviews on this subject are given by Leben (1965), Sinha (1965), and Last and Warren (1972).

Some plant pathogenic *Alternaria* spp. have been reported to be antagonized by certain saprophytic phylloplane fungi. Heuvel (1969) found that some isolates of *Aureobasidium pullulans* (de Bary) Arn. were antagonistic to *A. zinniae* Pape on *Phaseolus* bean leaves. Fokkema and Lorbeer (1974) reported that superficial mycelial development of *A. porri* (Ellis) Cif. on onion leaves was affected by *Sporobolomyces roseus* Kluijver & V. Niel, *Cladosporium herbarum* (Pers.) Link ex S.F. Gray and *A. pullulans*. Pace and Campbell (1974) isolated 38 fungal species from mature cabbage leaves and found that 11 of them, including *A. pullulans* and *Epicoccum nigrum* Link ex Fr., were antagonistic to *A. brassicicola* (Schw.) Wilt., in culture.

Mycoparasitism

Fungi parasitic on other fungi are termed "mycoparasites" and the term, mycoparasitism, indicates the relationship of a fungus parasite and a fungus host. Mycoparasitism was first recognized by de Bary (1865) who noticed *Piptocephalis freseniana* de Bary and *Cicinnobolus cesati* de Bary to be parasitic on other fungi. The mode of parasitism by *P. freseniana* was later studied by Brefeld (1872). These pioneer studies were followed by numerous reports describing mycoparasitism of different host-parasite combinations. Almost all taxonomic groups of fungi are now included in the list of mycoparasites and in some cases both host and parasite belong to the same genus and even to the same species. Furthermore, self-parasitism (parasitism within the same clone of a fungus) has also been reported (Klebs, 1899; Leong, McKeen and Smith, 1969; Nolan, 1975). However, relatively few species of mycoparasites have been studied intensively and positive demonstrations of a parasitic relationship are lacking in most associations reported. In fact, Barnett and Binder (1973) pointed out that a great majority of fungi observed growing on other fungi in nature may be merely fungicolous fungi. Fungicolous fungi are defined as those which live on other fungi without parasitic interactions (Gilman and Tiffany, 1952). True mycoparasites belonging to the major groups of fungi were reviewed and their mode of parasitism was discussed by Madelin (1968).

The descriptive terminology of mycoparasites has been inconsistent; however, the most widely used terms are "biotrophic" and "necrotrophic" (Gäumann, 1946). Biotrophic parasites are defined as those which are able to obtain nutrients from the living host cell but cause little or no damage to the host. Necrotrophic parasites are those which kill the host cells and acquire nutrients from the dead cells (Barnett, 1963 and 1964; Boosalis, 1964; Barnett and Binder, 1973). The former and latter groups are also called balanced and destructive parasites, respectively. Generally, a high degree of host specificity exists within the biotrophic group and a parasite may be restricted to a single host species or to a few closely related species. Unlike the biotrophic parasites, the necrotrophic parasites have a relatively broad host range. A majority of these fungi are thought to excrete toxic substances or enzymes which bring about quick killing of host cells. They are capable of growing on dead fungal structures as well as on common laboratory media.

The biotrophic (balanced) mycoparasites are further divided into three groups based on the mode of parasitism: (1) the internal parasites; (2) the contact parasites; and (3) the haustorial parasites (Barnett and Binder, 1973). The first group is represented by the chytrids, including species of *Rozella* Cornu and *Olpidium* (Braun) Rabenh., which develop a zoosporangium or a resting spore within cells of other fungi (Karling, 1942).

The contact parasites do not produce any internal structures within the host cells. *Gonatobotrys simplex* Corda is a good example of this group. This fungus was found to be associated with *Alternaria* species in nature, and parasitized *Alternaria* spp., *Cladosporium* spp. and *Paecilomyces* sp. in culture (Whaley and Barnett, 1963). The parasite produced specialized, short bulbous to finger-like, contact cells which did not penetrate the host cells. Whaley and Barnett (1963) speculated that the contact cells absorb nutrients by increasing the permeability of the host cell membrane to the nutrients required by the parasite. However, recently Hoch (1977a) carried out an electron microscopic observation of *G. simplex* - *A. tenuis* C.G. Nees host-parasite interface and suggested that the parasite obtains nutrients from the host through cytoplasmic continuity, via plasmodesmata.

Mycelium of *G. simplex* transferred to fresh culture media, failed to grow in axenic culture; however, water extracts from mycelium of hosts and many non-hosts were effective in promoting axenic growth of *G. simplex* (Whaley and Barnett, 1963). The partially purified growth factor was effective in very low concentrations and term "mycotrophein" was proposed for the vitamin-like substance. A similar growth factor was reported by Barnett and Lilly (1958) for *Calcarisporium parasiticum* Barnett, which is also a biotrophic contact mycoparasite.

Piptocephalis species are the most intensively studied biotrophic haustorial mycoparasites (Brefeld, 1872; Dobbs and English, 1954; Berry and Barnett, 1957; Armentrout and Wilson, 1969; Manocha and Lee, 1971; Manocha, 1975; Jeffries and Young, 1976). Species of *Piptocephalis* de Bary parasitize only Mucorales, except for *P. xenophila* Dobbs & English, which can parasitize several species of Ascomycetes and Imperfects (Dobbs and English, 1954). Axenic culturing of *Piptocephalis* has not been successful (Barnett and Binder, 1973). Berry and Barnett (1957) studied the mode of parasitism by *P. virginiana* Lead. & Mercer and reported that germ tubes of this parasite showed a strong tropic response toward a nearby host hypha. The hypha was penetrated by means of a hyphal peg and a haustorium was then formed inside the host. Manocha and Lee (1971) conducted an electron microscopy study of the host-parasite interface between *P. virginiana* and *Choanephora cucurbitarum* (Berk. & Rav.) Thaxter, and suggested that penetration of the host cell by the parasite involves mechanical forces. At the penetration site, the host cell wall extended inward and formed a collar around the haustorial neck. The mature haustorium was surrounded by an electron-dense sheath which appeared to be composed of cell wall material. Manocha and Lee (1971) speculated that formation of a new cell wall restricts further branching and growth of the haustorium and reduces the metabolic interchange between host and parasite. The presence of an electron-dense

sheath around the haustorial wall was also found at the *Mycotypha microspora* Fenner - *P. virginiana* host-parasite interface (Armentrout and Wilson, 1969). In this case, however, no collar was formed around the haustorial neck.

Abnormal cell growth of host hyphae is, in some cases, induced by biotrophic haustorial mycoparasites. England (1969) showed that when a young hypha of *C. cucurbitarum* was severely parasitized by *P. virginiana*, it responded by a proliferation of lateral branching resembling a witches' broom. *Dispira simplex* Benjamin has been reported to cause an enlargement of the host cells that contain haustoria (Brunk and Barnett, 1966). Besides these fungi, *Syncephalis* spp., *Dimargaris* sp., and *Tieghemiomyces* sp., all belonging to Mucorales, have been categorized as biotrophic haustorial mycoparasites (Barnett and Binder, 1973).

The necrotrophic (destructive) mycoparasites have received relatively little detailed study with the exception of *Trichoderma* spp. (Weindling, 1932; Boosalis, 1956; Hashioka and Fukita, 1969; Dennis and Webster, 1971; Hashioka, 1973; Komatsu, 1976) and *Gliocladium roseum* (Link) Bain (Shigo, 1958; Barnett and Lilly, 1962; Ricard, Grosclaude and Ale-agha, 1974; Walker and Maude, 1975). Weindling (1932) was first to recognize that *T. lignorum* (Tode) Harz is parasitic on other soil fungi. In culture, the parasite killed the aerial hyphae of *Rhizoctonia solani* Kühn by coiling around or

by breaking them at the septa. Submerged hyphae of the host died and broke up in regions in advance of the invading parasite. This observation provided strong circumstantial evidence of the production of diffusible and destructive enzymes or toxins by the parasite. The taxonomic disposition of Weindling's isolate, however, was later questioned by Webster and Lomas (1964). They re-examined the isolate and concluded that it was not *T. lignorum* but *G. virens* Miller, Giddens & Foster. Komatsu (1976) reported that *T. harzianum* Rifai and *T. polysporum* (Link ex Pers.) Rifai coiled extensively around hyphae of *Lentinus edodes* (Berk.) Sing. and destroyed the host colony in culture. When mycelia of *L. edodes* were soaked in culture filtrates of the *Trichoderma* spp., the cytoplasm of the host hyphae became coagulated and the contents exuded from the hyphal tips. Hashioka and Fukita (1969) carried out electron microscopy studies on the parasitism of some phytopathogenic fungi by *T. longibrachiatum* Rifai and *T. polysporum*. The mycoparasites formed neither appressorium nor haustorium, but induced infection papillae in the affected host cell wall.

Scytalidium album Beyer & Kling. is another example of necrotrophic mycoparasites which produce highly diffusible toxic substances. Klingström and Johansson (1972) demonstrated that after incubation with *S. album*, wood blocks contained a toxic factor which inhibited the growth of several decay fungi, e.g., *Fomes annosus* (Fr.) Cke. and *Polyporus versicolor*

L. ex Fr. Some strains of *S. lignicolum* Pesante, however, killed the decay fungi without producing significant concentrations of toxic substances, indicating a different antagonistic mechanism.

Barnett and Lilly (1962) compared the destructive activities of *G. roseum* on five host fungi and revealed that no fungus tested was immune to attack at all stages of development. *Ceratocystis fimbriata* Ell. & Halst. was the most susceptible host. The parasite, growing near host hyphae, was often stimulated to produce lateral branches that were attracted directly to the host cells (positive tropism), and killing of spores and vegetative cells occurred only following contact by the parasite. Barnett and Lilly (1962) thus concluded that enzymes or toxic substances responsible for the death of the host cells are active only near the parasite hyphae and do not diffuse into the medium for any observable distance. Similar conclusions were reached by Warren (1948) and by Butler (1957) who studied other necrotrophic parasites, *Papulospora* sp. and *R. solani*, respectively. Walker and Maude (1975), however, observed that *G. roseum* produced diffusible substances which were inhibitory to the growth of *Botrytis allii* Munn in culture, and in mixtures of spores of the 2 fungi those of *B. allii* failed to germinate.

The perfect state of *G. roseum* is *N. gliocladioides* Smalley & Hansen (Barnett and Lilly, 1962) and this is the only species of *Nectria* which has been studied intensively for its

mycoparasitic ability. *Nectria inventa* had not been reported as a mycoparasite prior to the present study.

Several species of *Verticillium* have been reported as mycoparasites. *Verticillium malthousei* Ware and *V. psalliotae* Treschow are parasites of the commercial mushroom (Treschow, 1941; Forer, Wuest, and Wagner, 1974). *Verticillium psalliotae* also parasitizes *Rhopalomyces elegans* Corda (Dayal and Barron, 1970). Barron and Fletcher (1970) demonstrated that *V. dahliae* Kleb and *V. albo-atrum* Reinke & Berth., two important soil-borne pathogens of higher plants, parasitized *R. elegans*. Both parasites readily penetrated conidiophores of the host, but evidence was not presented to show that vegetative hyphae were infected. Locci, Ferrante, and Rodrigues (1971) described the ultrastructure of the mode of mycoparasitism of the coffee rust, *Hemileia vastatrix* Berk. & Br. by *V. hemileiae* Bour.

A number of other fungi, including several common wood-rotting Basidiomycetes (Griffith and Barnett, 1967), have also been described as necrotrophic mycoparasites. However, little is known about the host-parasite relationship of these fungi.

More examples of both the necrotrophic and the biotrophic types as well as physiological aspects of mycoparasitism have been given and discussed in several review articles (Barnett, 1963 and 1964; Boosalis, 1964; Barnett and Binder, 1973).

Biological Control

Biological control has been defined as "the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonist" (Baker and Cook, 1974). There are numerous reports on biological control of phytopathogenic fungi by saprophytic or less virulent fungal isolates (e.g., Wood, 1951; Warren, 1972; Fokkema, 1973; Grosclaude, Ricard, and Dabos, 1973; Backman and Kabana, 1975; Mower, Snyder, and Hancock, 1975; Kelley, 1976; Huang, 1977). A number of examples of biological control, and probable mechanisms, have been reviewed by Garrett (1955), Baker (1968), Mitchell (1973), Papavizas (1973), Wilhelm (1973), Baker and Cook (1974), Fokkema (1976), and Skidmore (1976b).

GENERAL MATERIALS AND METHODS

Cultivars of rapeseed used in this study were Midas (*Brassica napus*) and Torch (*B. campestris*). Seeds of these varieties were supplied by the Experimental Farm of the University of Alberta.

Alternaria brassicae used in this study was isolated by K.J. Degenhardt, Department of Plant Science, University of Alberta, from a blackspot stem lesion on rapeseed grown in Edmonton, Alberta. Identification of this isolate was confirmed

by G.A. Petrie, Canada Department of Agriculture Research Station, Saskatoon, Saskatchewan.

Nectria inventa was isolated from a leaf surface of rapeseed during the present investigation and was identified by the Commonwealth Mycological Institute (IMI 189322).

Both *A. brassicae* and *N. inventa* were deposited at the American Type Culture Collection. The accession numbers assigned to the respective fungi are ATCC 34642 and ATCC 34641.

The cultures of *A. brassicae* and *N. inventa* were maintained on V-8 juice agar (V-8 juice, 200 ml; $CaCO_3$, 3 g; agar, 20 g per liter of distilled water) containing rose bengal (50 mg), and Difco potato-dextrose agar (PDA), respectively, at 25°C in the dark.

SECTION I

SOME PHYLLOPLANE FUNGI OF RAPESEED AND THEIR INTERACTIONS WITH *ALTERNARIA BRASSICAE*

A. ISOLATION OF PHYLLOPLANE FUNGI OF RAPESEED

INTRODUCTION

In this investigation, isolation of phylloplane fungi of rapeseed was conducted as a first step in a study to determine whether any of them could act as an antagonist of *Alternaria brassicae*.

MATERIALS AND METHODS

A plot of 13 m square, located at the Experimental Farm of the University of Alberta and removed from other rapeseed crops by about 50 m was used for this experiment. This plot was divided into 2 sub-plots (each 13 m x 5 m) with a 3 m wide open area between them. Each plot was sown with a different cultivar, Midas or Torch, on June 4, 1974. There were approximately 25 rows per plot, 13 m long, and about 20 cm apart.

Leaves were collected on June 25th and at 2-week intervals thereafter until September 3rd. Plants for leaf sampling were selected on the diagonal in each plot. Positions of sampled leaves of each collection are given in Table II.

Three different methods were used to isolate fungi from the leaf surfaces: (1) Three disks (18 mm in diameter) per leaf were cut with a cork borer from the upper, middle, and lower parts of 30 leaves for each cultivar. The cork borer was sterilized before each cutting by dipping in alcohol and flaming. Each batch of three disks from a leaf was shaken for 10 minutes in 18 ml of sterile distilled water in a 200 ml flask. One ml of the wash water or 1 ml of the wash water diluted by 10 times with sterile distilled water were plated out on PDA containing streptomycin sulfate (50 mg per liter); (2) The washed leaf disks from (1) were further shaken in 2 changes of 18 ml of sterile distilled water for 10 minutes per washing. After washing, free water on the disks was removed using sterile filter paper. Each batch of 3 disks was then placed on 2% water agar containing streptomycin sulfate (50 mg per liter); and (3) Three disks per leaf were cut from a second set of 30 leaves for each cultivar as in method (1) and were placed, without washing, on sterile filter paper which was placed in a sterile plastic petri dish (8.5 cm in diameter). The filter paper was moistened with 2 ml of sterile distilled water containing streptomycin sulfate (50 mg per liter). The petri dishes were placed in polyethylene bags to prevent desiccation. After incubation at room temperature for 10-14 days in the case of methods (1) and (2) and for 14-20 days for method (3), the fungi present were recorded and subcultures were made

where required. These 3 methods were used for the leaves collected on the 21st of June (the first sampling) and only method (2) was employed thereafter.

Straws of rapeseed remaining on the soil of the same plots were collected on May 15th, 1975. The straws were cut into pieces, about 5 cm long, and were washed thoroughly with tap water and later with sterile distilled water. After drying on sterile filter paper at room temperature, the straws were kept in sterile plastic petri dishes lined with moistened filter paper. After 14-20 days of incubation at room temperature, fungi which developed on the straws were recorded.

For identification of fungi, keys and descriptions given by the following were used: Ainsworth, Sparrow, and Sussman, 1973a,b; Ames, 1961; Barnett, 1972; Barron, 1968; Booth, 1959 and 1971; Dennis, 1968; Ellis, 1971; Hughes, 1951; Petch, 1938; Rifai, 1969; Seth, 1970; De Vries, 1967.

Myxomycetes, true yeasts, *Albugo candida* (Hook.) O. Kuntze and *Peronospora parasitica* (Pers. ex Fr.) Fr. were excluded from this study.

RESULTS

Methods (1) and (3) were not suitable for isolating pure cultures of different kinds of phylloplane fungi. Only those fungi which sporulated abundantly were isolated by method (1), and these included mostly *Cladosporium herbarum*, *Alternaria alternata* (Fr.) Keissler and *Penicillium* spp. Pure cultures were difficult to obtain by method (3) because of contamination. Moreover *Chaetomium* spp. (mainly *C. olivaceum* Cooke & Ellis) and *Nectria inventa* appeared to be stimulated to grow rapidly in the presence of filter paper and thus overgrew other fungi. In contrast, pure cultures of various phylloplane fungi were readily obtained by method (2) and, therefore, this method was employed throughout this experiment.

All identified genera isolated by this method are listed in Table I. These fungi occurred in three main groups: (1) Those which occurred rarely throughout the growing season of rapeseed; (2) Those which developed mostly after the onset of senescence of leaves (approximately 9th week for Torch and 11th week for Midas); and (3) Those which were isolated commonly throughout the growth stages of rapeseed. Fungi belonging to the group (1) included *A. raphani*, *Aspergillus* spp., *Aureobasidium* sp., *Botrytis* sp., *Cercospora* sp., *Doratomyces* spp., *Humicola* sp., *Mucor* sp., *Trichoderma polysporum*, and *Verticillium* sp. The fungi occurring mostly on senescent leaves (group 2) were: *Arthrobotrys* sp.,

Cephalosporium sp., *Coprinus* sp., *Dendryphion* sp., *Gilmaniella humicola* Barron, *Scopulariopsis* sp., *Stachybotris* sp., *Stigmina* sp., and *Torula herbarum* (Pers.) Link ex Gray. *Arthrobotrys* sp., *Dendryphion* sp., and *Stigmina* sp. were found only on Torch.

Seasonal variation in the occurrence of selected fungi is listed in Table II. These fungi belonged to group (3) with the exception of *A. raphani* (group (1)) and *Drechslera* sp., *T. harzianum*, *Trichothecium roseum* Link and *Phoma* sp., which were intermediate between groups (1) and (3). Among these fungi, *A. alternata*, *Cladosporium* spp. (mainly *C. herbarum*), and *Fusarium* spp. (mainly *F. avenaceum* (Corda ex Fr.) Sacc.) were dominant at any growth stage of both Midas and Torch. *Nectria inventa* also occurred in high frequency especially in August and September on both cultivars. This fungus together with *A. alternata* and *C. herbarum* also dominated the flora on the dead straws of rapeseed.

The causal fungi of blackspot of rapeseed, *A. brassicae* and *A. raphani*, occurred infrequently. The former appeared on both Midas and Torch, but mostly in the later stages of plant development. The latter was isolated at a very low frequency, and only from Torch.

Besides the fungi mentioned above, there were several isolates which were unidentified. Most of these fungi did not sporulate on the culture media used.

TABLE I. Genera of phylloplane fungi isolated from Midas and Torch cultivars of rapeseed

Phycomycetes
- Mucor Mich. ex Fr.
- Rhizopus Ehrenb. ex Corda

Ascomycetes
- Chaetomium Kunze ex Fr.
- Nectria Fr.[1] (conidial)
- Pleospora Rabenh. ex Ces & de Not.

Basidiomycete
- Coprinus (Pers. ex Fr.) Gray[2]

Fungi Imperfecti
- Alternaria Nees ex Fr.
- Arthrobotrys Corda[2,3]
- Aspergillus Mich. ex Fr.
- Aureobasidium Viala & Boyer
- Botrytis Pers. ex Fr.
- Cephalosporium Corda
- Cercospora Fres.
- Cladosporium Link ex Fr.
- Dendryphion Wallr.[2,3]
- Doratomyces Corda
- Drechslera Ito
- Fusarium Link ex Fr.
- Gilmaniella Barron[2]
- Gliocladium Corda
- Humicola Traaen
- Paecilomyces Bain
- Penicillium Link ex Fr.
- Phoma sensu Sacc.
- Scopulariopsis Bain
- Stachybotris Corda[2]
- Stemphylium Wallr.
- Stigmina Sacc.[2,3]
- Torula Pers. ex Fr.[2]
- Trichoderma Pers. ex Fr.
- Trichothecium Link ex Fr.
- Ulocladium Preuss
- Verticillium Nees ex Wallr.

[1] *Verticillium* state.

[2] Genera isolated mostly from senescent leaves.

[3] Genera isolated only from the cultivar Torch. Other genera were found on both cultivars.

TABLE II. Seasonal variations in the occurrence of major phylloplane fungi on two cultivars of rapeseed

Sampling date	June 25		July 9		July 23		August 6		August 20		September 3	
Weeks after seeding	3		5		7		9		11		13	
Cultivar of rapeseed[1]	M	T	M	T	M	T	M	T	M	T[8]	M	T
Sampled leaves[2]	2	2-3	3-4	4-5	4-5	6-8	5-6	9-10	6-8	-	8-9	-
Alternaria alternata	30[3]	26	27	25	30	27	30	30	30	-	30	-
A. brassicae	0	0	1	0	0	0	6	6	4	-	5	-
A. raphani	0	0	0	2	0	0	0	1	0	-	0	-
Chaetomium spp.[4]	0	2	3	3	1	3	3	0	0	-	0	-
Cladosporium spp.[5]	29	28	30	30	28	29	30	29	30	-	30	-
Drechslera sp.[6]	0	2	0	0	0	0	0	6	3	-	0	-
Fusarium spp.[7]	25	27	27	28	17	27	29	29	21	-	13	-
Nectria inventa	3	7	2	4	0	5	12	16	24	-	27	-
Penicillium spp.	4	2	3	1	0	0	3	0	4	-	0	-
Phoma sp.	0	1	0	1	2	1	1	2	2	-	1	-
Pleospora sp.	3	2	2	8	5	9	12	3	7	-	6	-
Rhizopus oryzae	0	0	5	3	2	0	4	1	0	-	0	-
Stemphylium sp.	0	0	0	0	3	2	1	3	6	-	10	-
Trichoderma harzianum	0	1	0	0	2	2	0	0	0	-	0	-
Trichothecium roseum	0	1	0	0	0	1	2	4	2	-	2	-
Ulocladium spp.	2	2	6	3	5	6	4	12	8	-	6	-

[1] M = Midas, T = Torch
[2] Position of sampled true leaves.
[3] Number of leaves (out of 30) from which the fungus was isolated. One leaf is represented by 3 disks made from the leaf.
[4] Mainly *C. olivaceum*.
[5] Mainly *C. herbarum*.
[6] *D. poae* (Baudys) Shoemaker like.
[7] Mainly *F. avenaceum*.
[8] No data due to defoliation.

DISCUSSION

The method used for isolating fungi from the phylloplane throughout the present investigation has certain advantages. By using leaf disks, one can always obtain the same leaf area (about 7.63 cm^2 per 3 disks) from each leaf. Leaf disks are easier to handle and they can be washed more thoroughly than a whole leaf. Water agar, due to its low nutrient content, results in relatively sparse colony development around each leaf disk, and thus makes it easier to obtain pure fungal cultures. It is also possible that this limited vegetative growth is accompanied by less intense microbial competition and hence a greater variety of fungi can develop. However, different media favor different fungi and, therefore, it is likely that there were some phylloplane fungi which could not grow on water agar and thus were overlooked in this study.

Although the addition of surface-active agents such as Tween 80 is known to improve the removal of leaf surface propagules (Dickinson, 1966), it is not yet certain whether they are nontoxic to any fungus. Therefore, any such agent was not used in this study. As a result, it is possible that some of the fungi recorded in Tables I and II (especially those of group (1)) are so-called "casuals" (Leben, 1965) which were on the sampled leaves by accident.

Fungi in group (2) are considered to have an active role in the decomposition of rapeseed leaves but are inactive until leaves become senescent. These fungi, therefore, are

not likely to be significant participants in microbial interactions on healthy leaves.

Species of *Cladosporium* (mainly *C. herbarum*) and *A. alternata*, which were the most commonly isolated phylloplane fungi in this experiment, have been shown to be the common phylloplane fungi of various plants and to be among the first colonizers of leaves (Webster, 1957; Meredith, 1962; Dickinson, 1966; Norse, 1972; Mishra and Srivastava, 1974). It may be assumed that these fungi are most likely to interact with other phylloplane fungi, including the blackspot pathogens.

Cladosporium herbarum and *A. alternata* also dominated the mycoflora developed on the rapeseed straws. This indicates their importance in the decomposition of rapeseed material in nature. Vaartnou, Tewari, and Horricks (1974) also found *C. herbarum* to be one of the dominant fungi on stems and roots of mature rapeseed.

Alternaria alternata was reported to be parasitic on Polish-type rapeseed in Alberta (Vaartnou and Tewari, 1972). In spite of its abundance on rapeseed leaves, however, characteristic symptoms described by these authors were not observed on the sampled leaves in this investigation. This suggests that *A. alternata* on rapeseed includes pathogenic as well as saprophytic strains as Vaartnou and Tewari (1972) had suggested.

Fusarium spp. (mainly *F. avenaceum*) and *N. inventa* are ranked next to *C. herbarum* and *A. alternata* in the frequency of occurrence. *Fusarium avenaceum* has virtually a world-wide distribution wherever crops are grown. It is a soil fungus and a prominent parasite of the Gramineae (Booth, 1971).

Among the fungi belonging to group (3), *T. harzianum* (Komatsu, 1976) and *T. roseum* (Barnett and Lilly, 1962) are well known as mycoparasites. The majority of the fungi in group (3) were examined for their antagonistic abilities against *A. brassicae* in Part B.

B. INTERACTIONS OF MAJOR PHYLLOPLANE FUNGI WITH *ALTERNARIA BRASSICAE* IN CULTURE

INTRODUCTION

The purpose of this study was to examine antagonistic abilities of the major phylloplane fungi of two rapeseed cultivars to *Alternaria brassicae*.

MATERIALS AND METHODS

All *Alternaria* species and 10 other fungi which were common in the phylloplane of rapeseed were chosen for this study. These fungi are listed in Table III.

Cultures of all the *Alternaria* species were maintained on V-8 juice agar with rose bengal, and others on PDA at 25°C in the dark. Two-week-old cultures were used throughout the experiment.

Antagonism of each test fungus to *A. brassicae* was examined by making dual cultures, inoculated 5 cm apart on PDA in 9 cm petri dishes. Four replicates were made for each of the 12 different combinations. Dual inoculated cultures of *A. brassicae* served as control. These cultures were incubated at 20°C in the dark and the final assessments of colony interactions were made after 2 weeks of incubation. A key given by Skidmore and Dickinson (1976) was used for these assessments. They developed a key based on the observations of Porter (1924) who recognized 5 different types of colony interactions:

(A) Mutually intermingling growth, where both fungi grew into one another without any macroscopic signs of interaction.

(Bi) Intermingling growth where the fungus being observed is growing into the opposite fungus either above or below or above and below its colony and its corollary.

(Bii) Intermingling growth where the fungus under observation has ceased growth and is being overgrown by another colony.

(C) Slight inhibition where the fungi approached each other until almost in contact and a narrow demarcation line, about 1-2 mm, between the two colonies was clearly visible.

(D) Mutual inhibition at a distance of more than 2 mm.

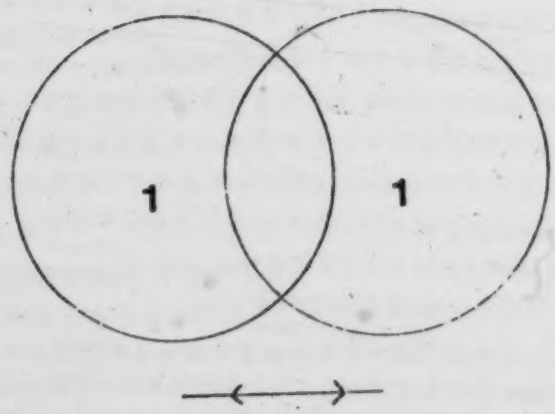
The interacting fungi were assigned values on a 0-5 scale for each type of interaction (Fig. 1).

Small squares of agar were cut from hyphal tip regions of *A. brassicae* colonies or from areas of intermingling growth. These agar blocks were mounted in water or in cotton blue-lactophenol on microscope slides, and the type of interactions and cytological changes of *Alternaria* cells, if any, were observed with a light microscope.

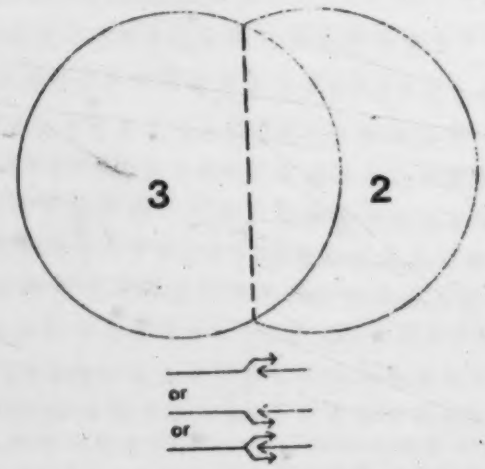
FIG. 1

Key used to assess colony interactions in culture
after A.M. Skidmore and C.H. Dickinson (1976)

A. Mutually intermingling growth

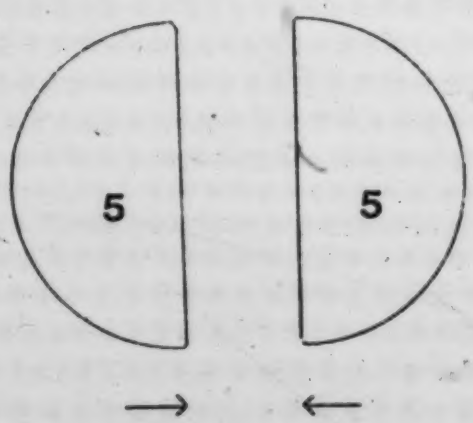


B. Overgrowth of antagonist



C. Mutual slight inhibition

D. Mutual inhibition at a distance



RESULTS

The types of interactions occurring between *A. brassicae* and other rapeseed phylloplane fungi are summarized in Table III. A narrow demarcation line (about 1-2 mm wide) developed and only a few hyphae of either fungus were present in the area between the colonies of *A. brassicae* and each of the following fungi: *A. alternata*, *A. brassicae* (control), *A. raphani*, *C. herbarum*, *Fusarium avenaceum*, and *Phoma* sp. These interactions were classified as type C (Fig. 1). Mutual inhibition at a distance (type D) was evident in the interactions between *A. brassicae* and two other fungi, *Drechslera* sp. and *Stemphylium* sp. A clear zone of 1.3-1.4 cm and 1.5-2.0 cm wide developed between the colonies of respective combinations. In the interactions of type C and D, hyphae of *A. brassicae* did not exhibit any visible cytological abnormalities.

Fungi which overgrew the colony of *A. brassicae* were *Chaetomium olivaceum*, *Nectria inventa*, *Rhizopus oryzae* Went & Geerligs, *Trichoderma harzianum*, and *Trichothecium roseum*. Among these fungi, only *R. oryzae* did not appear to exert any detrimental effect on *A. brassicae* hyphae, although the growth of the latter appeared to cease when the two colonies met. The other 4 fungi were parasitic to *A. brassicae*. Among them, *C. olivaceum*, *N. inventa*, and *T. roseum* appeared to be similar in the mode of parasitism. The

growth of *A. brassicae* ceased when hyphae of these fungi were in close proximity. Hyphae of these parasites often coiled around *Alternaria* hyphae. At the sites where contact was made, formation of swollen appressorium-like bodies was frequent in *N. inventa* and *T. roseum* but rare in *C. olivaceum*. Cells of *A. brassicae*, particularly those of conidia, were often penetrated by these parasites. Cytological abnormalities in the host cells induced by these parasites were also similar. Host cells which were in contact with the parasite cells commonly showed a somewhat granular appearance when stained with cotton blue. The appressorium-like bodies of *N. inventa* and *T. roseum* usually induced the formation of a reaction zone (the region between the cell wall and the invaginated plasma membrane developed in the host cell. See Section II.A for detail) in the host cells. Vacuolation of the host cells occasionally occurred in the later stages of parasitism and the cells eventually appeared to be empty. Despite the similarities in the mode of parasitism, however, *N. inventa* was the most destructive. Wherever *Nectria* hyphae came into contact, intact host cells were seldom found.

Trichoderma harzianum attacked *A. brassicae* in a different way. The growth of *A. brassicae* appeared to cease at a considerable distance from *Trichoderma* hyphae. Extensive vacuolation occurred in the cells of host conidia, as well as in the hyphae, without physical contact. Coiling of the host

TABLE III. Interactions between *Alternaria brassicae* and some other phylloplane fungi on potato-dextrose agar medium

Test Fungi	Colony interactions (Scores)[2]	Type of abnormalities appearing in the cells of *A. brassicae*[3]	Mode of parasitism[4]	Index of susceptibility of *A. brassicae*[5]
Alternaria alternata	4	-		I
A. raphani	4	-		I
Chaetomium olivaceum	3	g,v,e	CO,*AP^0,P	S
Cladosporium herbarum	4	-		I
Drechslera sp.	5	-		I
Fusarium avenaceum	4	-		I
Nectria inventa	3	g,*v^0,r,e	CO,AP,P	VS
Phoma sp.	4	-		I
Rhizopus oryzae	3	-		I
Stemphylium sp.	5	-		I
Trichoderma harzianum	3	g,v,e	CO	VS
Trichothecium roseum	3	g,v^0,r,e	CO,AP,P	R-S
Control[1]	4	-		I

[1] Dual culture of *A. brassicae*.
[2] From Fig. 1.
[3] g = granulation, v = vacuolation, r = reaction zone, e = emptiness, - = no visible abnormality
[4] CO = coiling, AP = appressorium-like bodies, P = penetration,
*small ^{0} indicates rare occurrence of the phenomenon.
[5] I = immune: no parasitic relation.
R = resistant: infected cells distributed sparsely in the intermingling area.
S = susceptible: infected cells abundant.
VS = very susceptible: more intense destruction than S.

hyphae by *Trichoderma* hyphae was common. The coiled host hyphae appeared to be empty, but, whether the killing occurred after physical contact was made or prior to that event could not be determined. Formation of appressorium-like bodies and penetration were not observed.

DISCUSSION

Alternaria brassicae had mutually inhibitory relations with *Drechslera* sp. and *Stemphylium* sp. Although it is not a major concern of this study to discuss the mechanisms of antagonism, one can speculate that in addition to competition for such substances which are limited in PDA and required by both fungi, some fungistatic metabolic products were possibly involved in these interactions. The fungistatic principle(s) could be either highly diffusible through PDA or volatile (Hutchinson, 1971; Fries, 1973). However, the absence of visible cytological abnormalities in *Alternaria* cells in dual cultures of these combinations suggests that these fungi coexist in the phylloplane of rapeseed without any obvious harm to each other.

Trichoderma harzianum also inhibited the growth of *A. brassicae* at a considerable distance, however, unlike *Drechslera* sp. and *Stemphylium* sp., *Trichoderma* caused a drastic degeneration of *Alternaria* cells. Whether this interaction between *T. harzianum* and *A. brassicæ* is parasitism is debatable. The term "parasitism" has been customarily used to indicate an association whereby one organism feeds on

another living organism. In this sense, in the stage where *Trichoderma* exerts a degenerative effect on *Alternaria* cells without physical contact, this association should be categorized as antibiosis. If the degenerated cells are dead when *Trichoderma* comes in contact, the acquisition of nutrients by *Trichoderma* from these cells, if any, is merely saprophytic. However, if *Trichoderma* cells can cause leakage of endogenous nutrients from *Alternaria* cells while they are still alive, and can absorb the nutrients directly through the host cell wall or through agar, this interaction may be called parasitism. Despite its strong antagonistic ability, however, the fact that *T. harzianum* rarely occurs on rapeseed leaves, precludes its importance in suppressing *A. brassicae* in the phylloplane.

Nectria inventa was isolated at a high frequency and showed the strongest parasitism to *A. brassicae*. Moreover, its mode of parasitism appeared to represent that of other fungi which were also parasitic to *A. brassicae*. Therefore, the *A. brassicae* - *N. inventa* combination was chosen as a model system for detailed studies on mycoparasitism and biological control.

SECTION II

ALTERNARIA BRASSICAE - *NECTRIA INVENTA* HOST-PARASITE INTERACTION

A. MODE OF PARASITISM OF *ALTERNARIA BRASSICAE* BY *NECTRIA INVENTA*

INTRODUCTION

Scanning or transmission electron microscopy has been used only rarely to demonstrate mycoparasitic interactions (e.g., Armentrout and Wilson, 1969; Hashioka and Fukita, 1969; Manocha and Lee, 1971; Hashioka, 1973; Jeffries and Yong, 1975; Carling, Brown, and Millikan, 1976; Ikediugwu, 1976a,b; Hoch, 1977a,b). However, these techniques are essential to obtain an overall picture of host-parasite interactions. Therefore, this study was conducted to observe the interaction, especially the host-parasite interface, between *Alternaria brassicae* and *Nectria inventa* with the aid of electron and light microscopy.

MATERIALS AND METHODS

Conidia of *A. brassicae* and *N. inventa* were collected by adding sterile distilled water to a 10- to 14-day-old culture and scraping the culture surface with an inoculation needle. The conidia were washed in a Büchner funnel and suspended in sterile distilled water. The spore density was adjusted to about 5×10^5 spores/ml for *A. brassicae* and to 10^8 spores/ml for *N. inventa*. Equal volumes of each spore suspension were mixed, and 0.5 ml of the mixture was spread evenly on a cellophane membrane disk (8-cm diameter) that was placed on 2% water agar in a plastic petri dish. The culture was incubated at 20°C in the dark for 24-72 hours.

For observation with a light microscope, after incubation, the cellophane membrane was cut into strips of appropriate sizes, and mounted in water or in cotton blue-lactophenol on a microscope slide.

For transmission electron microscopy, the mycelia grown on the cellophane membrane were gently brushed off with a paint brush into 3% glutaraldehyde in 0.1M Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0). The liquid containing the mycelia was passed through a Millipore filter (pore size 0.45 µm). The mycelia retained on the filter were fixed overnight in glutaraldehyde, washed with buffer, and postfixed in 2% OsO_4 in the same buffer for 3-5 hours. The material was dehydrated in an ethanol series, taken to propylene oxide, and embedded

in Araldite 502. Gold and silver sections were cut on a Reichert Model Om-U2 ultratome with a glass or a diamond knife. The thin sections were stained with aqueous 2% uranylacetate for 2 hours followed by aqueous 0.2% lead citrate for 5 minutes. Electron micrographs were taken in Philips EM-300 and EM-200 electron microscopes.

Thin sections (about 1 μm) of the material embedded in Araldite were also made for light microscopic observation. These sections were stained with a slightly alkaline solution of toluidine blue (1%) in borax (1%) (Meek, 1970).

For scanning electron microscopy, a thin layer of water agar was made on a cover slip (15-mm diameter) and a drop of the mixed spore suspension was smeared over it. These spores were incubated for 24-72 hours in a plastic petri dish lined with moist filter paper. The mycelia that developed on the agar layer were fixed by the same procedures as for transmission electron microscopy. The fixed mycelia on agar were dried by either freeze drying or critical-point drying. In the former method, the fixed mycelia on agar were frozen quickly in Freon 22, stored briefly in liquid nitrogen and freeze-dried in vacuum at - - 70°C (Nei, 1974). The cover slips with freeze-dried material were stuck to stubs with conductive glue and coated with carbon and gold in a vacuum evaporator. In the case of critical-point drying, the fixed material was dehydrated in ethanol-water series and taken through absolute ethanol-amyl acetate series into amyl acetate.

The material was critical-point dried in a Denton vacuum DCP unit using carbon dioxide (Anderson, 1951), and shadowed twice with gold. Micrographs were taken in a Cambridge stereoscan S4 scanning electron microscope.

RESULTS

The colony of conidial *N. inventa* was characterized by its bright orange-red color. Phialides were produced in whorls on the primary conidiophore axis or branches thereof (Plate 1, A). Conidia were ovoid, smooth, and aggregated in masses at the tips of the phialides (Plate 1, B).

The average diameters of hyphae of *N. inventa* and *A. brassicae* grown on water agar were 1.0 µm and 3.5 µm, respectively. The hyphae and conidia of *A. brassicae* had a thick superficial electron-dense layer that either was absent or inconspicuous in *N. inventa*. The presence of lomasomes in *Nectria* hyphae and their absence, except in some parasitized cells of *A. brassicae*, was another characteristic that facilitated identification of the two fungi in thin sections.

Parasitism was evident after incubation for 24 hours, and by 96 hours, the majority of hyphae and conidia of *A. brassicae* were heavily parasitized. Parasitism occurred either by means of contact of the respective hyphae without penetration or by penetration of *A. brassicae* by *N. inventa*.

Parasitism through contact

Growth of *N. inventa* hyphae was more profuse in the vicinity of *A. brassicae* hyphae, and was particularly intense around conidia. The growth pattern of *N. inventa* hyphae around and on the hyphae of *A. brassicae* is illustrated by scanning electron micrographs (Plate 1, C-E) and light micrographs (Plate 2, A-D). Tropic growth of the parasite hyphae toward the host hyphae occurred when the former was in close proximity (up to about [illegible] µm) to the latter (Plate 1, C and Plate 2, A-C). The parasite hyphae either coiled around the *Alternaria* hyphae (Plate 1, D and E) or, more frequently, grew parallel and produced short aerial branches toward the host hyphae (Plate 2, A-C). The parasite hyphae appeared to come in contact with the *Alternaria* hyphae either by being appressed to them or, more commonly, by means of appressorium-like bodies that become attached to the host hyphae. Short branches which arose from the parasite hyphae usually became swollen upon contact with the host hyphae and formed the appressorium-like bodies (Plate 2, A and C).

Conidia and conidiophores of *A. brassicae* were parasitized in the same way as its vegetative hyphae. The parasite hyphae grew toward the conidium of *A. brassicae* (Plate 3) and entwined the host conidium. These parasite hyphae appeared to favor attachment to the septal area and to the basal portion of the germ tube (Plate 4, A). Formation of appressorium-like bodies was frequent. Scanning electron

micrographs revealed that a fibrous material was deposited at the point of contact of the appressorium-like bodies with the host conidium (Plate 4, B) and hypha (Plate 4, C). Eventually, the host conidia were entwined by a profuse growth of parasite hyphae that caused them to collapse (Plate 5, A and B).

An abnormal response of the host cell usually was evident at the point of contact by the parasite hypha, and was especially evident in the cells beneath the swollen appressorium-like bodies of *N. inventa*. In these areas the plasma membrane of the host cell had an invaginated outline (Plate 6). The reaction zone (the region between the cell wall and the invaginated plasma membrane) was largely electron-transparent, and usually contained dispersed electron-dense material that often was organized into tubule-like structures (Plate 6 and Plate 7, A-C). Early stages of infection generally showed the electron-dense material largely as tubules (Plate 7, B), while in the later stages it appeared as irregular deposits (Plate 7, D).

Reaction zones were also found in light micrographs (Plate 2, A and B) and at a certain stage of infection, plasmolysis of the host cytoplasm occurred as shown by staining with cotton blue-lactophenol (Plate 2, A and Plate 5, A).

Unusual accumulation of membranous material sometimes occurred in the affected cell (Plate 7, A). A septal plug often was formed between an invaded cell and an adjacent cell

(Plate 6 and Plate 7, A). In a few cases, lomasomes were present below the reaction zone (Plate 7, C).

In advanced stages of parasitism the plasma membrane became disorganized and eventually disappeared (Plate 6). The cellular organelles were disrupted and only remnants of some membranous elements and scattered ribosomes remained (Plate 7, D).

Nectria inventa hyphae apparently were not able to induce these abnormal responses in host cells at a distance.

Parasitism by means of penetration

Penetration of *Alternaria* hyphae by *Nectria* hyphae was rare, but penetration of conidia was common. Contact parasitism was usually effected by the formation of an appressorium-like body; however, penetration commonly occurred without forming it. Penetration of septa in the hyphae of *A. brassicae* resulted in a separation of cells (Plate 1, C). Penetration of a mature conidium also occurred commonly at septal areas (Plate 8, A and B and Plate 9, B and C), although sometimes it occurred at random locations on other parts of the spore surface.

The septum of a conidium, as revealed by transmission electron microscopy, appeared to be composed of 5 layers (Plate 8, B and C). The cell wall of a conidium consisted of at least 4 layers (C_1-C_4 in Plate 8, C). The middle electron-lucid layer of the septum was derived from the C_2 layer and was flanked on either side by an electron-dense

layer which was continuous with the C_3 layer. These electron-dense layers were thickened at the rim of the septum and formed a triangular junction of the cross wall and lateral wall. The outermost layers of the septum were electron-lucid and were continuous with a C_4 layer. These layers became almost electron-transparent in the area adjacent to the plasma membrane.

During the initial stages of penetration, the outer two layers of the conidial wall dissolved at the point of contact with the hyphal tip of *N. inventa* without showing any evidence of deformation of the fibrillar structure of the cell wall (Plate 9, A). When a conidium was penetrated at a septum, the parasite hyphae further degraded the triangular junction and in most cases it proceeded to grow intercellularly by separating the septal wall through its electron-lucid middle layer (Plate 9, B). A reaction zone usually developed at a penetration site (Plate 8, B and Plate 9, B); however, sometimes host cells appeared to have been penetrated without developing it.

At the penetration site, a large hole often developed in the host cell wall (Plate 8, B and Plate 11, B) and the presence of a meshwork of material in the hole was demonstrated with the aid of scanning electron microscopy (Plate 11, B).

An electron-transparent zone surrounded the internal parasite hypha (Plate 9, C). The internal hypha penetrated from cell to cell by the production of a swollen structure and

a constriction at the site of passage through the host cell wall (Plate 10, A and B). The cytoplasm of invaded host cells became progressively less dense. Later, only some lipid bodies, disorganized membranes, and scattered ribosomes remained. The host cells eventually appeared to be empty and the walls collapsed (Plate 10, A and B and Plate 11, A).

Juvenile conidia of *A. brassicae* were produced on conidiophores arising either from hyphae or directly from mature conidia. Usually they were invaded through the basal pore by *N. inventa* hyphae that reached this spot by growing inside the host conidiophore (Plate 11, C). The basal pore was demonstrated by scanning electron microscopy in the detached juvenile conidium (Plate 11, E). Inside the juvenile conidium, the parasite hyphae usually invaded other cells through septal pores (Plate 11, C) which appeared to be fully open (Plate 11, D).

Plate 1

Scanning electron micrographs of the *Verticillium* state of *Nectria inventa* and its growth pattern around and on the hyphae of *Alternaria brassicae*:

A) Phialides produced in whorls on conidiophores. X 850;

B) Masses of conidia on phialides. X 1500;

C) Parasite hyphae (P) attacking a host hypha (H). The parasite hyphae are penetrating the septa (arrows). X 2000;

D) and E) Parasite hyphae coiling around host hyphae. X 12000.

Legend: H = host; HB = hyphal branch of the host; P = parasite.

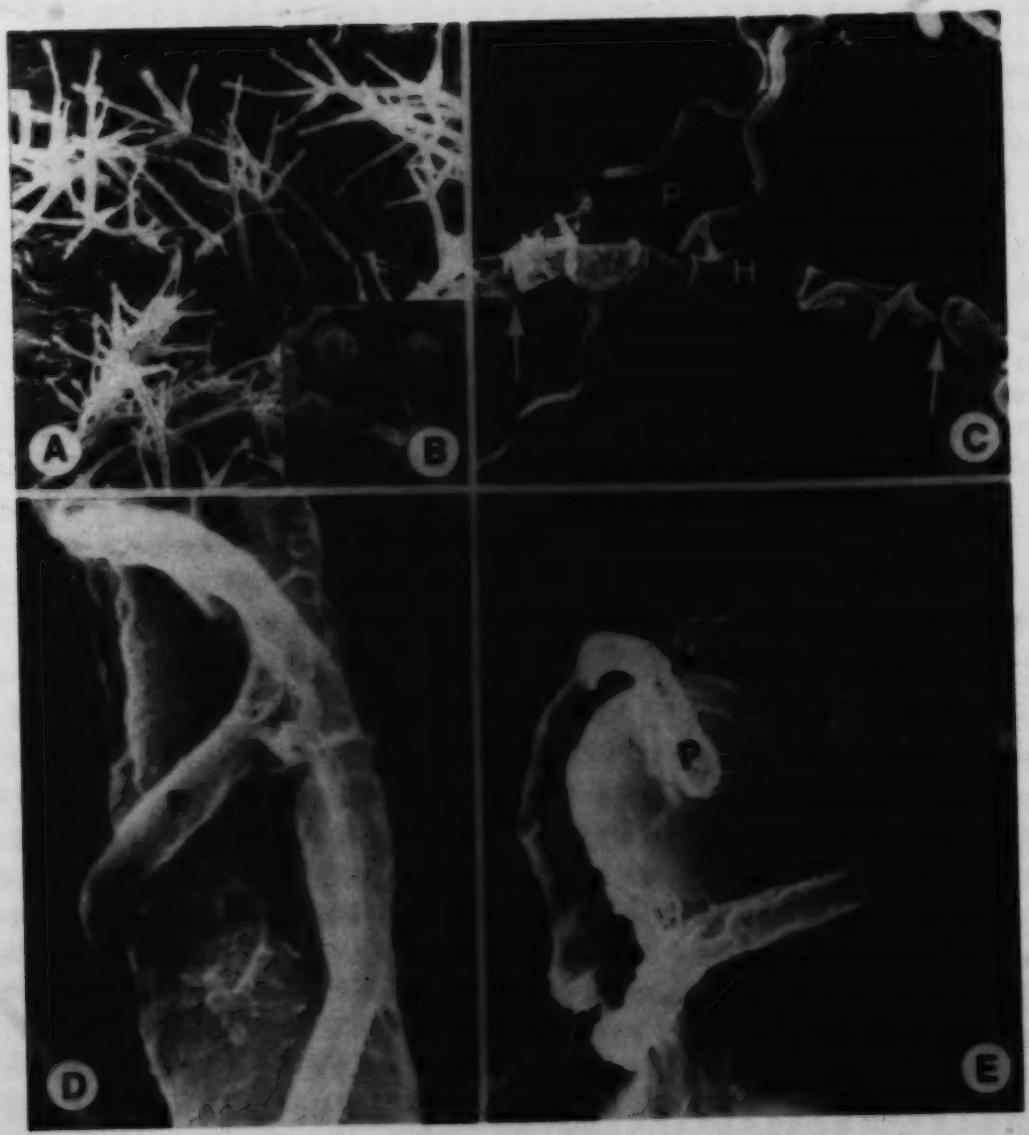

A
B
C
D
E

Plate 2

Light micrographs of hyphae of *Nectria inventa* parasitizing hyphae of *Alternaria brassicae*:

A) Parasite hyphae growing parallel to a host hypha. Note the swollen appressorium-like bodies of the parasite and cells of the host showing plasmolysis (phase-contrast). X 4300;

B) Parasite hypha causing reaction zones (arrows) in a host hypha. Host cell which has not been contacted by the parasite hypha appears to be healthy. X 1000;

C) Tropic growth of parasite hyphae toward host hyphae. X 400;

D) Late stage of parasitism. Host cells contacted by parasite hyphae appear to be empty. X 2100.

Legend: A = appressorium-like body; H = host; P = parasite.

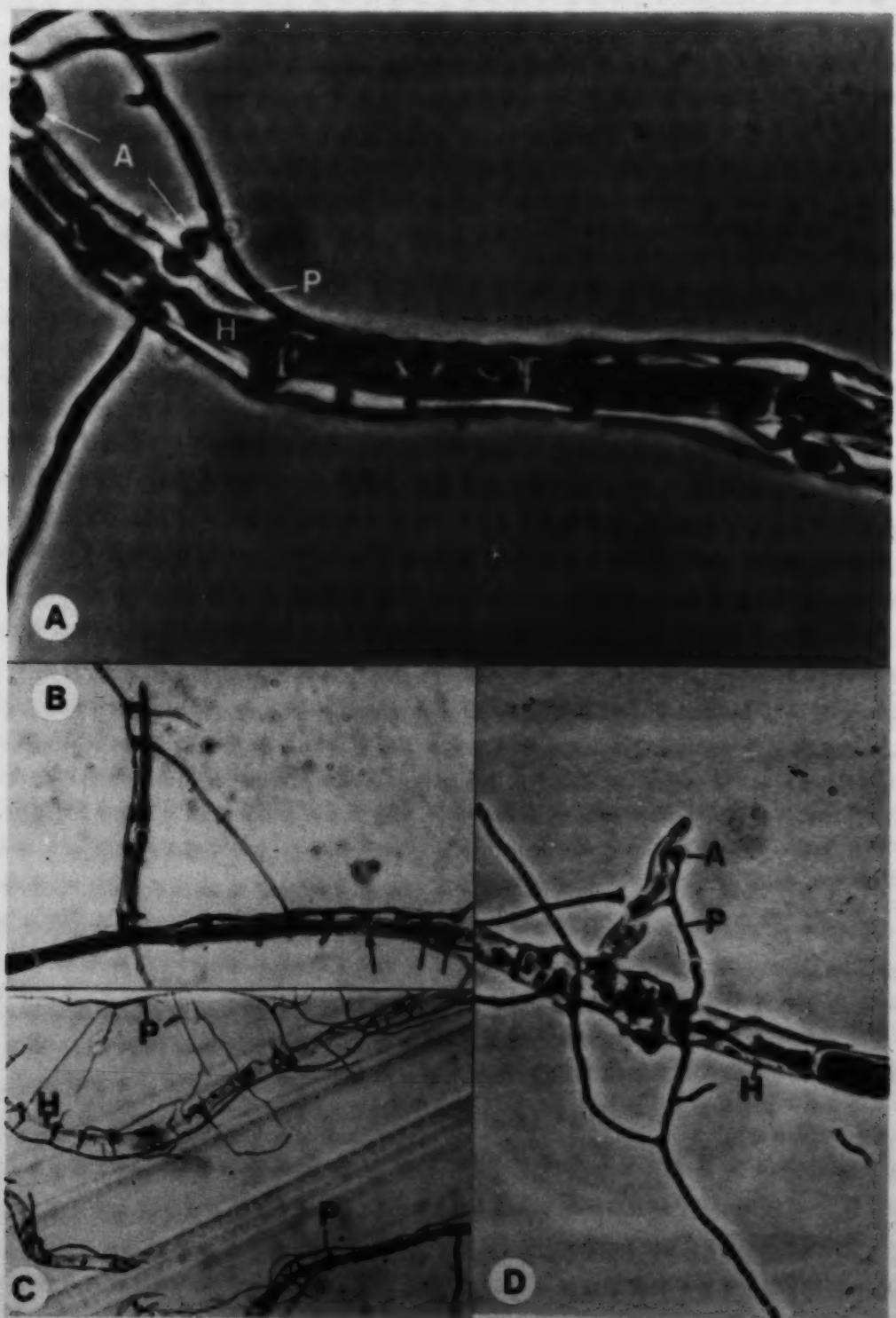

A
P
H
A
B
P
H
P
C
A
P
H
D

Plate 3

Tropic growth of short branches from a hypha of *Nectria inventa* toward a conidium of *Alternaria brassicae*. X 4500.

Legend: H = host; P = parasite.

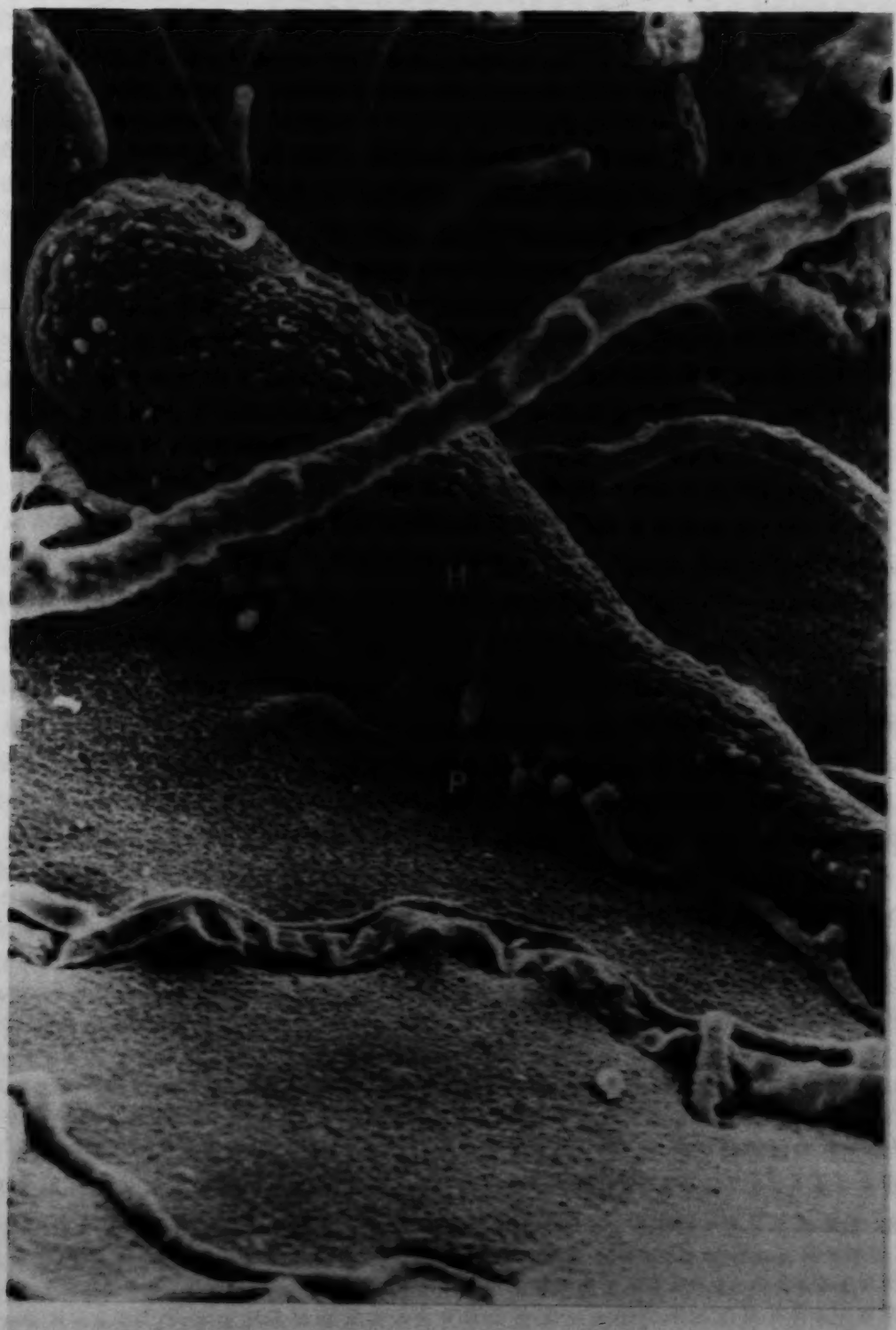


Plate 4

Scanning electron micrographs of parasite hyphae growing on *Alternaria brassicae* and appressorium-like bodies of the parasite:

A) Parasite hyphae occurring predominantly in the septal area (arrows) and the basal portions of the germ tubes of a host conidium. X 1800;

B) Appressorium-like bodies formed on the host conidium. Note presence of adhesive material under these bodies. X 11000;

C) Appressorium-like body with fibrous adhesive material formed on a host hypha. X 25000.

Legend: A = appressorium-like body; G = germ tube; H = host.

G
A
B
C

Plate 5

Phase-contrast light micrographs of conidia of *Alternaria brassicae* parasitized by *Nectria inventa*:

A) Healthy-appearing conidium and infected mature and juvenile conidia. Note a host cell showing plasmolysis and non-infected cells (arrows) in the heavily infected conidium. X 1200;

B) Profuse growth of parasite hyphae around a host conidium. X 1200.

Legend: C = host conidiophore; JC = juvenile host conidium; MC = mature host conidium; P = parasite.

JC
C
MC
P
A
B

Plate 6

Transmission electron micrograph of cells of *Alternaria brassicae* parasitized by *Nectria inventa* through contact: Development of reaction zone occurs in host hyphal cells in response to contact by the parasite. Note invagination of host plasma membrane, electron-dense tubule-like structures and deposits below the host cell wall, presence of septal plug, and lack of recognizable host plasma membrane (arrow) in the middle of the upper host cell. X 31500.

Legend: ED = electron-dense deposit; HCW = host cell wall; HP = host plasma membrane; M = mitochondrion; P = parasite; RZ = reaction zone; SP = septal plug; TS = tubule-like structures; W = Woronin body.

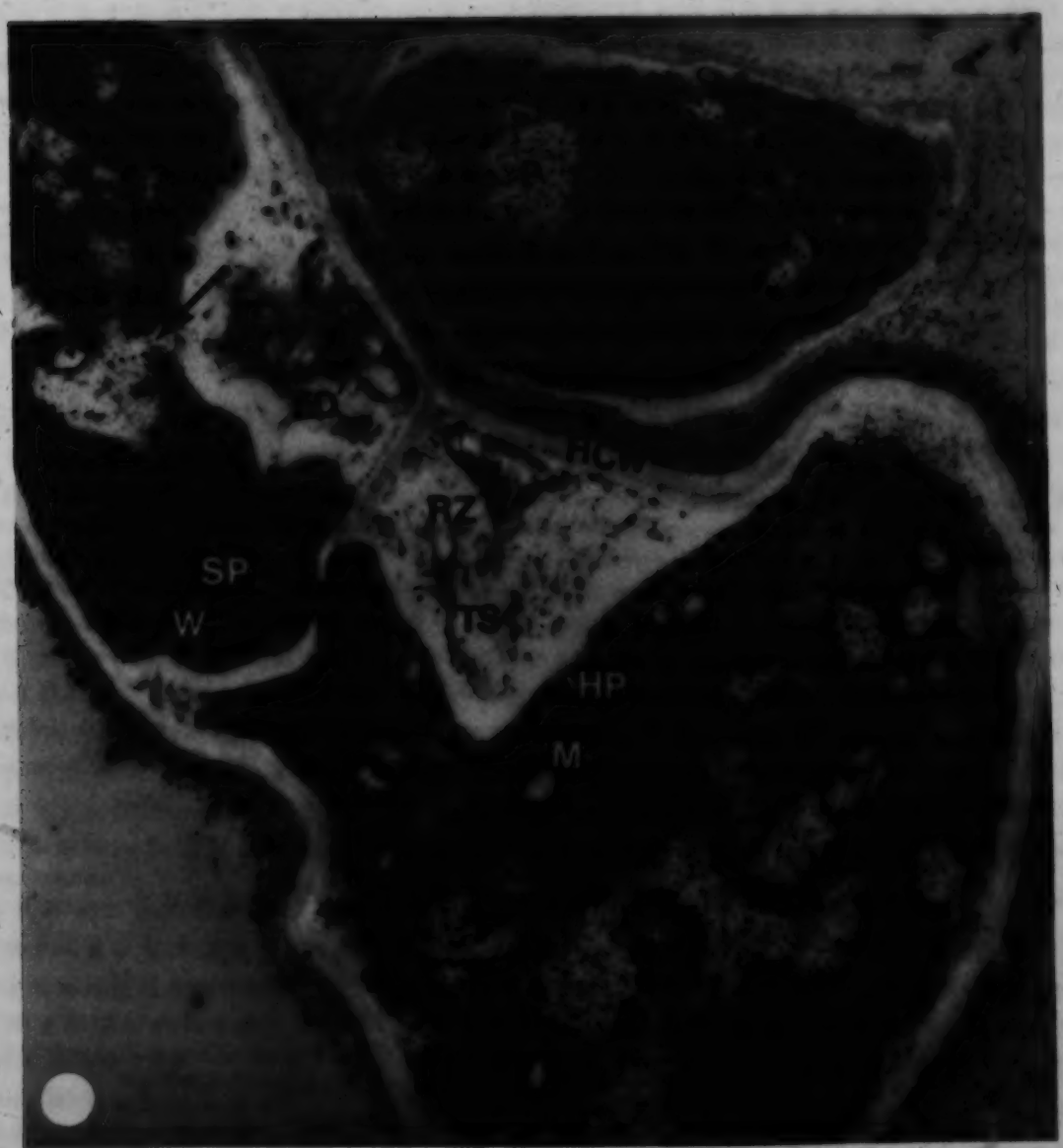

RZ
SP
W
TS
HP
M

Plate 7

Transmission electron micrographs of different stages of contact parasitism of *Alternaria brassicae* by *Nectria inventa*:

A) Unusual accumulation of membranes (arrow) in an infected host hyphal cell. X 38000;

B) Initial development of a reaction zone in host conidial cells. An extensive development of tubule-like structures is evident in the reaction zone. X 22000;

C) Lomasomes in the area adjacent to the reaction zone in a host conidial cell (arrow). X 43000;

D) Advanced stage of parasitism. Note lack of electron-dense deposit in one reaction zone (arrow) and disintegrated cytoplasm of the host hyphal cell. X 22000.

Legend: ED = electron-dense deposit; HCW = host cell wall; HP = host plasma membrane; L = lomasome; M = mitochondrion; N = nucleus; NO = nucleolus; RM = remnants of membranous material; RZ = reaction zone; SP = septal plug; TS = tubule-like structures.

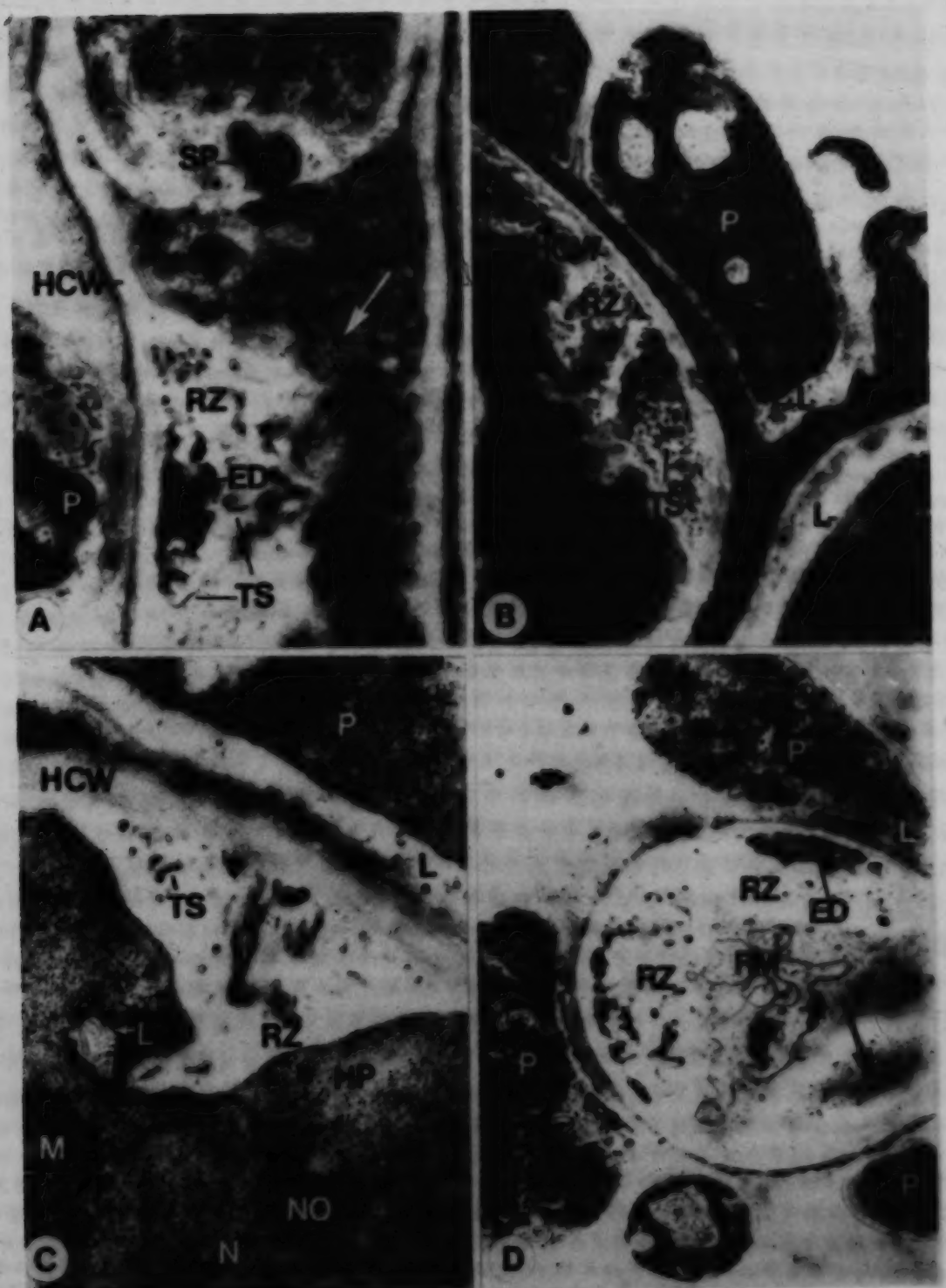

SP
HCW
RZ
ED
P
TS
A
P
RZ
L
B
HCW
P
L
TS
L
RZ
HP
M
NO
N
C
P
L
RZ
ED
RZ
P
P
D

Plate 8

Ultrastructure of a septum of *Alternaria* conidium and penetration by *Nectria inventa* at the septum:

A) and B) Light and transmission electron micrographs of thin sections showing penetration of conidia of *A. brassicae* by *N. inventa* at a septum. Note dissolution of the host cell wall and development of reaction zones in the host cells in B). A), X 2300. B), X 19000;

C) Ultrastructure of septal part of a normal conidium of *A. brassicae*. Cell wall is composed of at least 4 layers (C_1 - C_4), and 3 of them (C_2 - C_4) appear to form the septal wall. X 90000.

Legend: ML = electron-lucid middle layer of the septum; P = parasite; RZ = reaction zone; S = septum; T = triangular junction of the septal wall and lateral wall.

A
P
RZ
P
S
RZ
B
C4
C3
C2
C1
T
ML
C

Plate 9

Transmission electron micrographs showing different stages of penetration of *Alternaria* conidium by *Nectria inventa*:

A) Initial stage. Dissolution of the outer cell wall material and lack of stress in the microfibrillar region of host cell wall (arrow) are evident. X 43000;

B) Stage prior to penetration. X 22000;

C) Penetrated host conidial cell. Compare the density of cytoplasm of the penetrated and healthy cells. Note the electron-transparent area surrounding the penetrating hypha of the parasite and lack of significant indentation of the host cell wall at the site of penetration. X 19000.

Legend: HCW = host cell wall; L = lomasome; LB = lipid body; P = parasite; R = ribosomes, RZ = reaction zone.

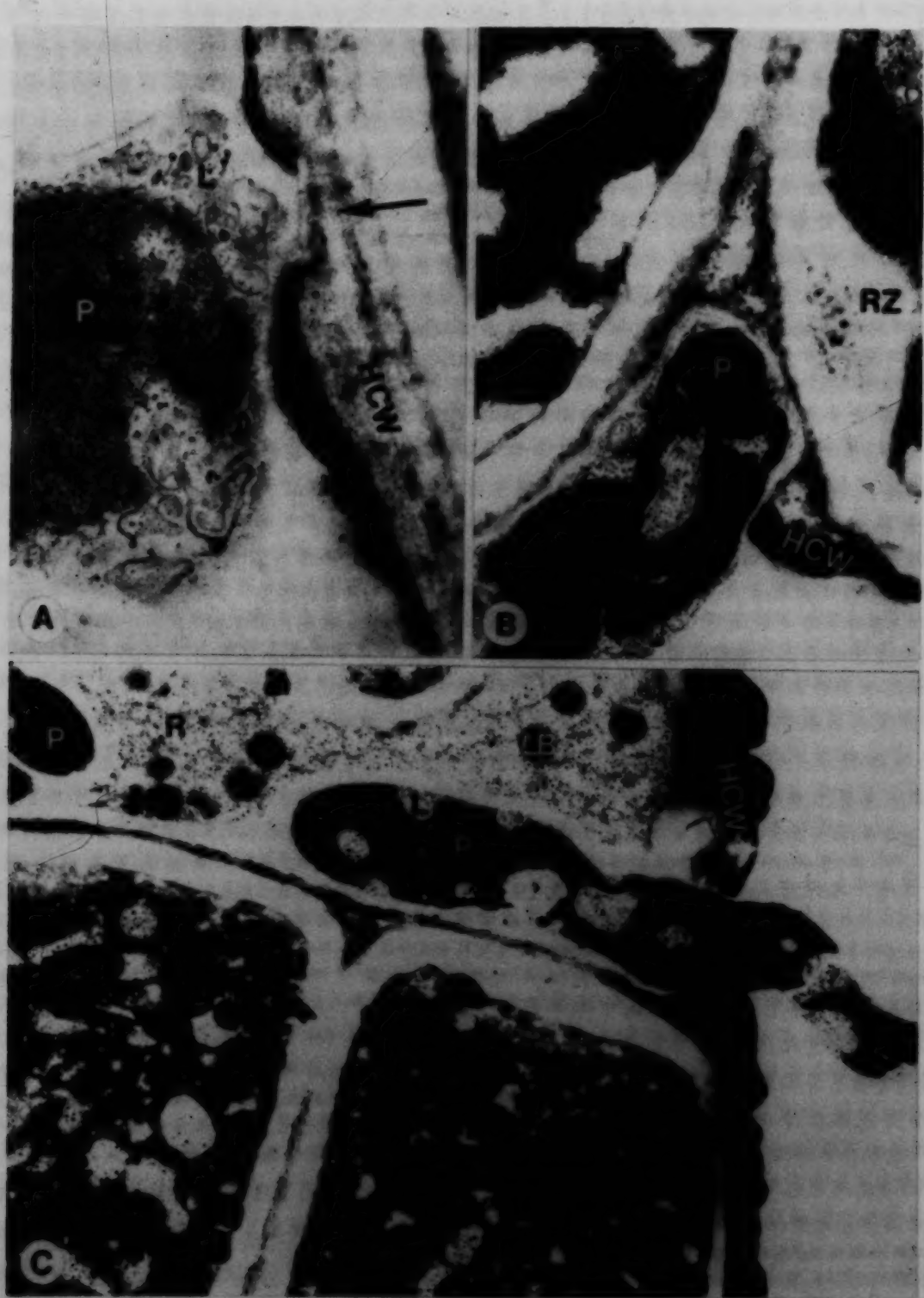

P
HCW
A
RZ
P
HCW
B
P
R
HCW
C

Plate 10

Hyphae of *Nectria inventa* growing inside the host conidium:

A) Swollen and constricted parasite hypha at the site of penetration. Penetrated cells appear to be empty and a partially degraded cell contains ruptured mitochondrion (arrow), remnants of other membranous organelles and ribosomes. X 20000;

B) Profuse growth of internal parasite hyphae. Note the degraded cell wall of the host conidium. X 2800;

Legend: CP = constriction of the parasite hypha; HC = host conidial cell; HCW = host cell wall; L = lomasome; P = parasite; S = swollen structure in the parasite hypha.

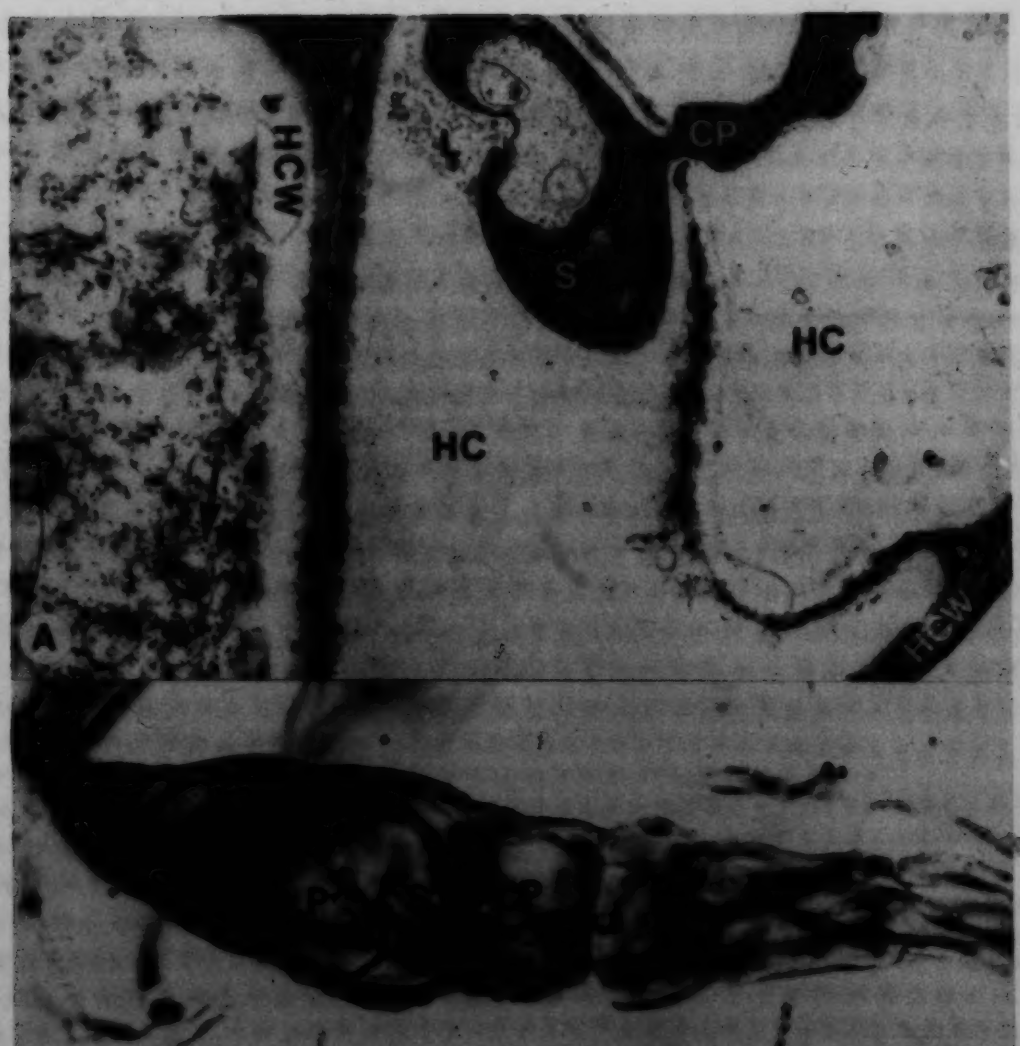

HCW
CP
S
HC
HC
HCW
A
B

Plate 11

Scanning electron and light micrographs showing penetration of mature and juvenile host conidia by *Nectria inventa*:

A) Mature conidium of *Alternaria brassicae* penetrated by hyphae of *N. inventa*. Note collapsed cell wall of the conidium (arrow). X 1800;

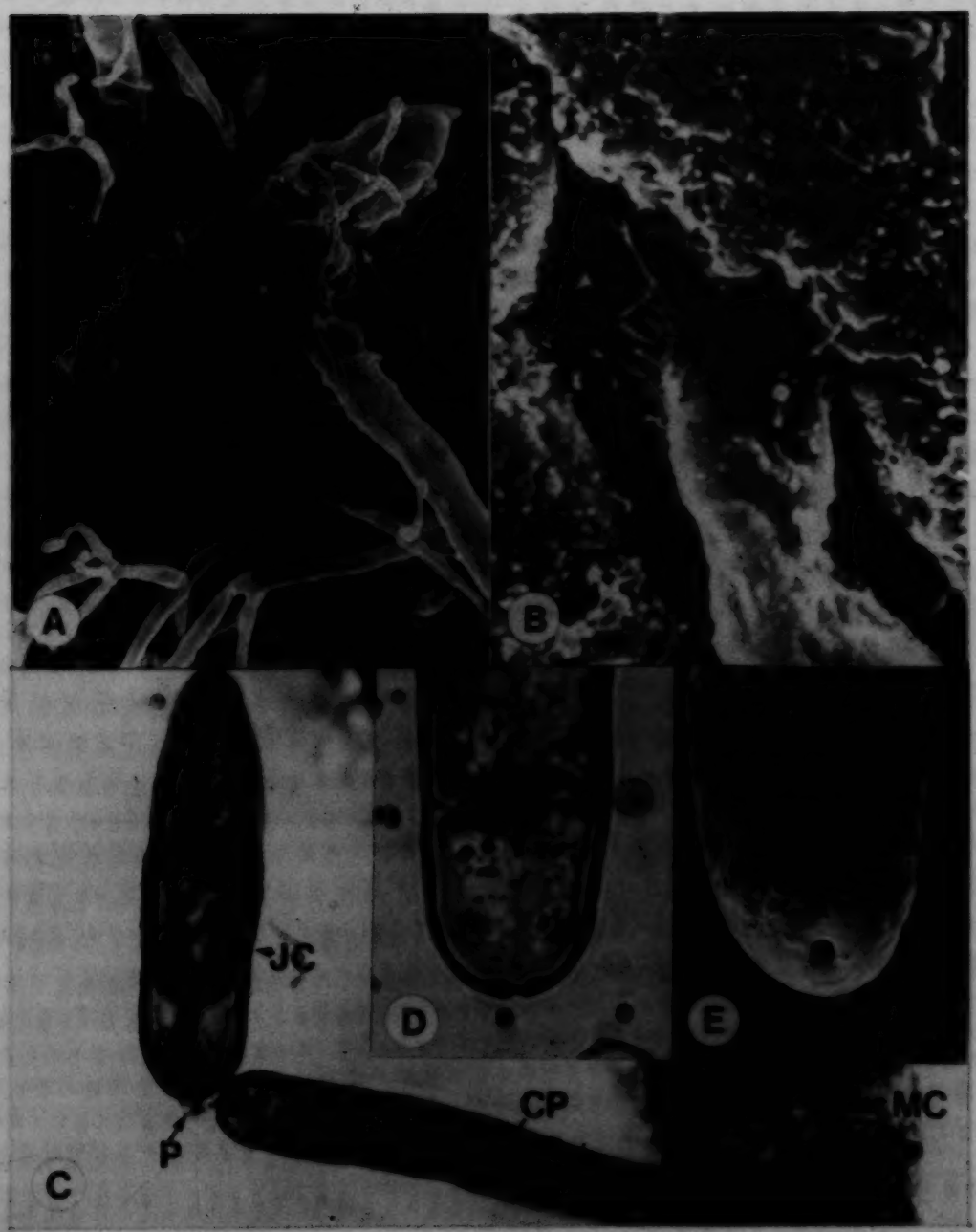
B) Enlarged view of a penetration site. A large hole develops in the wall of the host cell. A meshwork of material appears at the penetration site (arrow). X 27000;

C) Juvenile conidium of *A. brassicae* penetrated through the basal pore by *N. inventa*. X 2500;

D) Thin section of a normal juvenile conidium of *A. brassicae* showing the basal pore and septal pore (arrow). X 2800;

E) Scanning electron micrograph of a basal pore in a juvenile conidium of *A. brassicae*. X 3500.

Legend: CP = conidiophore produced by a mature conidium; JC = juvenile conidium; MC = infected cells of the mature conidium; P = parasite.


A
B
JC
D
E
CP
MC
P
C

DISCUSSION

In general, parasitism of *A. brassicae* by *N. inventa* appears to proceed in the following sequence:

(1) Tropic growth of *Nectria* hyphae toward *Alternaria* cells;

(2) Physical contact of the respective fungi;

(3) Formation of appressorium-like bodies and adhesive material by the parasite;

(4) Development of reaction zones in the host cells;

(5) Formation of septal plugs and in some cases an unusual membrane system in the host cells;

(6) Penetration of the host cells by *N. inventa* (rare in host hyphal cells); and

(7) Disintegration of the host cytoplasm and the cell wall.

The tropic phenomenon is common in mycoparasitism (Barnett and Lilly, 1962; Boosalis, 1964); however, factors which induce it are not well known. In the *A. brassicae* - *N. inventa* interaction, it is most likely that the host conidia and hyphae create specific microenvironments around them in such a way that the parasite hyphae can detect the presence of the host at a distance, and respond by initiating the tropic growth. The differences in microenvironment, e.g., temperature, moisture, pH, composition of nutrients, may be caused by various activities of the host such as respiration, and exudation or leakage of endogenous material (see Section II. C).

This kind of special micro-area may exist around almost any fungus, thus resembling the concept of the rhizosphere in higher plants. Therefore, this area is proposed to be called a "mycosphere". This concept is further supported by the occurrence of exudates containing various materials from sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary (Colotelo, 1973) and also from hyphae of a number of fungi (Personal communication with Dr. N. Colotelo).

Several mycoparasites have been reported to form appressoria in contact with their host cells (Buller, 1934; Berry and Barnett, 1957; Benjamin, 1959; Jeffries and Young, 1975). Species of *Piptocephalis* are the most studied examples where, during infection of the host hyphae, appressoria are formed, followed by penetration with fine infection pegs, and then formation of branched haustoria (Berry and Barnett, 1957; Benjamin, 1959). Since the swollen bodies formed by *N. inventa* infrequently penetrate *Alternaria* hyphae, therefore they should be called appressorium-like bodies.

The presence of an adhesive material has been reported not only in various fungus - higher plant interactions (Edwards and Allen, 1970; Bracker and Littlefield, 1973; Endo and Colt, 1974) but also at the *Puccinia graminis* Pers. - *Darluca filum* (Biv. Bern. ex Fr.) Cast. host-parasite interface (Carling, Brown, and Millikan, 1976). The adhesive

material also usually occurs at the *A. brassicae* - *N. inventa* interface and it appears to be produced by the parasite (Plate 4, B and C).

The appearance of the reaction zone that usually develops in the host cells in response to contact by *N. inventa* hyphae is somewhat similar to a papilla. Papillae are commonly formed in cells of vascular plants in response to penetration by pathogenic fungi, such as *Erysiphe* sp. (Edwards and Allen, 1970; McKeen and Rimmer, 1973). Edwards and Allen (1970) described papillae as electron-dense with membranous material embedded in an amorphous matrix. Bushnell and Bergquist (1975) suggested that papillae are significant components of generalized host resistance to powdery mildew fungi. Similarly, in myco-parasitic associations, Butler (1957) observed deposition of wall-like material around infection hyphae of *Rhizoctonia solani* in invaded cells of some phycomycetous fungi such as *Rhizopus nigricans* Ehreb. Recently, Swart (1975) demonstrated that wall thickenings were induced in cells of *Phycomyces blackesleeanus* Burgeff and *Aspergillus clavatus* Desmazieres as a result of parasitism by *Verticillium dahliae*. The term "callosity" was used for the wall thickenings. Formation of callosity was confined to sporangiophores or conidiophores of the host fungi and was not detected in the vegetative hyphae. Manocha and Lee (1971), however, reported that papillae were not detected in cells of *Choanephora cucurbitarum* penetrated by *Piptocephalis virginiana*.

The exact mechanism which induces the reaction zone in *Alternaria* cells is not known; however, it is speculated that enzyme(s) or toxic substance(s) released by *N. inventa* are involved in this phenomenon. This speculation is supported by the following works on host specific toxins produced by 2 phytopathogenic fungi. Park and his co-workers (1976) found that AK-toxin produced by *A. kikuchiana* Tanaka causes an immediate increase in permeability and ultrastructural changes in susceptible cells in the vascular bundle sheath and in the mesophyll of Japanese pear. The first obvious change in the ultrastructure was an invagination of the plasma membrane, evident within 1 hour after exposure. Leaf cells became necrotic after 10 hours of toxin exposure and the spaces between susceptible cell walls and invaginated membranes contained many lomasome-like vesicles, membrane fragments and darkly stained materials. Victorin, a host specific toxin, produced by *Helminthosporium victoriae* Meehan & Murphy is also known to cause almost identical changes in the susceptible host cells (Luke, Warmke, and Hanchey, 1966; Samaddar and Scheffer, 1971). These changes are strikingly similar to those which occurred during the formation of reaction zones in the *Alternaria* cells in response to *N. inventa*.

The occurrence of plasmolysis of the infected *Alternaria* cells (Plate 2, A and Plate 5, A) indicates that a change in permeability occurs in the host cells when infected by *N. inventa*. This appears to be a strong indication of the bidirectional flow of materials through the host-parasite interface; enzyme(s) and/or toxic substance(s) flow from the parasite and endogenous nutrients from the host. Thus, *N. inventa* is able to acquire nutrients from *Alternaria* cells which are most likely still alive. The toxic substance(s), if any, may not be highly diffusible through agar or it may be produced only in response to contact with host cells because *N. inventa* does not cause any visible abnormalities in the *Alternaria* cells at any visible distance. Its primary mode of action is most likely on the host membrane systems that control permeability. In fact, the plasma membrane of *A. brassicae* appears to be ruptured during the attack by *N. inventa* (Plate 6). Barnett (1964) also suggested that the biotrophic contact mycoparasites, *Calcarisporium parasiticum* and *Gonatobotrys simplex*, may absorb nutrients from the living host by altering the permeability of the cell membrane.

In certain higher plant-fungal parasite combinations, fungal penetrations commonly incite the formation of wall-like deposits on the inner surfaces of host walls, and the wall thickenings have often been considered as host cell wall ingrowths (Aist, 1976). However, this is not the case for

the electron-dense amorphous deposits of *A. brassicae*; these deposits consist of degradation products from tubule-like structures, that often accumulate beneath the host cell wall in the early stages of infection. Transmission electron micrographs (Plate 6 and Plate 7, A-D) reveal that the innermost host cell wall layer is not so affected by the contact of *N. inventa*. The electron density of this layer obviously differs from that of the deposits, suggesting that they are chemically different.

The origin of the wall deposits in infected plant cells, i.e., whether the material is deposited by the host protoplast or by the fungus, has long been debated among plant pathologists (Aist, 1976). The results of this study indicate that the electron-dense deposits of *A. brassicae* are primarily of host origin. Neither the tubule-like structures nor the electron-dense deposits appear to be produced after the vitality of the invaded *Alternaria* cells declines (Plate 7, D).

Abnormal membrane production occurs commonly in vascular plants when infected by pathogenic fungi (Peyton and Bowen, 1963; Hess, 1969; Edwards and Allen, 1970). According to Whaley *et al.* (1964), it also can be induced in onion cells by abiotic stresses such as mechanical injury. The abnormal membrane production that is evident sometimes in invaded cells of *Alternaria*, however, can be regarded as a host response to *Nectria*. This response, as well as the

formation of electron-dense irregular deposits, in some way may be resistant reactions of the host cells to penetration by *N. inventa* hyphae.

The function of lomasomes, often observed in cells of the parasite at the host-parasite interface, is not well understood; they may participate in absorptive or secretory functions of the parasite hyphae (Peyton and Bowen, 1963).

Formation of septal plugs generally regarded as a protective mechanism may also be a host resistance response.

Although Manocha and Lee (1971) suggested that penetration of *C. cucurbitarum* by *P. virginiana* primarily may involve a mechanical process, this may not be a major mechanism for penetration of *A. brassicae* cells by *N. inventa* hyphae; instead it appears to be primarily chemical in nature. This conclusion is based on the following observations:

(1) The micrograph showing an initial stage of penetration (Plate 9, A) indicates that there is little or no stress in the microfibrillar portion of the host cell wall beneath the tip of a parasite hypha, and there is no significant indentation of the host cell wall at the site of penetration (Plate 9, B and C);

(2) A large hole in the host cell wall is often evident around the penetrating parasite hypha;

(3) A meshwork of material occurs at the penetration site and it appears to be the product of enzymatic breakdown of host cell wall material; and

(4) In older infections there is a distinct disintegration of the conidial cell wall.

This is further supported by the fact that *N. inventa* has strong extracellular enzymatic activities such as that of β-glucosidase (Personal communication with Dr. N. Colotelo).

Penetration of a host conidium occurs commonly at a septum. It is likely that after the enzymatic degradation of the three layers of cell wall (C_1, C_2, and C_3 in Plate 8, C), the parasite hypha can readily penetrate intercellularly (Plate 8, B). It subsequently degrades the remaining wall layer to complete the penetration.

A problem arises when one attempts to describe *N. inventa*. Is it biotrophic or necrotrophic? As discussed before, the ability of this parasite either to upset the permeability control of host cells or to penetrate healthy-appearing host cells, indicates that the parasite is most likely able to obtain nutrients from living host cells. It would thus be biotrophic in nature at least in the early stage of parasitism. However, with regard to ultrastructural changes of infected host cells, the effect of *N. inventa* is not balanced but destructive. It differs considerably from that of biotrophic (balanced) parasites which usually do not destroy the ultrastructural integrity of the host cells and establish a harmonious relationship with the host cells (Armentrout and Wilson, 1969; Manocha and Lee, 1971; Jeffries and Young, 1976; Hoch, 1977a, b). In higher plant cells, enrichment of the host cytoplasm with ribosomes,

dictyosomes, mitochondria, and endoplasmic reticulum often occurs in response to biotrophic (balanced) fungal parasites (Ehrlich and Ehrlich, 1963; Berlin and Bowen, 1964). Therefore, *N. inventa* is best described as a destructive or necrotrophic mycoparasite.

B. FORMATION OF MICROSCLEROTIA AND CHLAMYDOSPORES FROM CONIDIA OF *ALTERNARIA BRASSICAE*, AND THEIR SUSCEPTIBILITY TO *NECTRIA INVENTA*

INTRODUCTION

In the previous part, the mode of parasitism of conidia and hyphae of *Alternaria brassicae* by *Nectria inventa* was described. In addition to conidia and hyphae, however, *A. brassicae* was found to form seemingly survival structures, microsclerotia and chlamydospores, from cells of conidia under certain cultural conditions. Therefore, the present study was conducted to describe the formation of these structures and to examine their susceptibility to *N. inventa*.

Petrie (1972) reported that the primary inoculum of *A. brassicae* consists of wind-borne spores produced on debris of rapeseed or certain cruciferous weeds. However, information is lacking on the means by which the pathogen overwinters and persists under adverse conditions.

Among species of *Alternaria*, Elliott (1917) noted that secondary growth in conidia occurs commonly in old cultures of several species of *Alternaria*, including *A. brassicae*. Conidia of *A. chlamydospora* Mouchacca, isolated from desert soil, also become very variable in shape because of their secondary growth (Ellis, 1976). Only one species, *A. padwickii* (Ganguly) Ellis has been reported to form sclerotia in culture (Ellis, 1971). Chlamydospores are produced in several species. For example, *A. raphani* forms

chlamydospores abundantly in culture (Groves and Skolko, 1944; Ellis 1971). Atkinson (1953) suggested that the survival of *A. raphani* in dry soil cultures for a long period (5 years) is related to the formation of these chlamydospores. Dark brown, aggregated chlamydospores are sometimes produced by *A. longissima* on natural and artificial substrata (Ellis, 1971). Chlamydospores of these species are formed by the rounding up of a cell or cells of a hypha. Basu (1971) noted that when normal conidia of *A. porri* (Ellis) Cif. f. sp. *solani* (Ell. & Mart.) are placed in natural soil, round, thick-walled chlamydospores form within single cells of the conidia.

MATERIALS AND METHODS

Rapeseed leaves infected by *A. brassicae*, and in a condition ranging from green to partially decayed, were collected from fields around Edmonton, Alberta. They were stored at 3°C in the dark and conidia of *A. brassicae* which formed on them were observed periodically with a Leitz Wetzlar microscope equipped with an Ultropak incident-light illuminator.

For studies of *A. brassicae* in culture, V-8 juice agar and V-8 juice agar with rose bengal at a concentration of 50 mg per liter were used. Cultures were incubated at 25°C in the dark for 2 weeks and half of them were transferred to 3°C to determine the effect of a low temperature. The cultures were kept in a polyethylene bag to prevent

desiccation. To study the effect of gradual desiccation on the formation of microsclerotia and chlamydospores, agar disks (5-cm diameter) were removed from 2-week-old cultures and kept in a sterile plastic petri dish at room temperature. Conidia, removed periodically from these cultures, were mounted in cotton blue-lactophenol on a microscope slide and examined with a light microscope.

Germination of microsclerotia was examined on a 1% malt-extract solution placed on a sterile microscope slide. The effect of freeze-thawing on the germination of microsclerotia was determined after they were stored at about -40°C for 2 months and then thawed at 3°C for 5 days.

Loam soil (black chernozemic silt loam soil developed on lacustrine), collected from a rapeseed plot at the Experimental Farm of the University of Alberta, was used for observing the response of *Alternaria* conidia to incubation on natural soil. The soil was sieved, its moisture content was adjusted to 25% weight/weight by adding sterile distilled water, and then placed in a sterile plastic petri dish. Conidia collected from a 2-week-old culture of *A. brassicae*, were washed in a Büchner funnel and suspended in sterile distilled water. A drop of the conidial suspension containing about 5×10^5 conidia per ml was dispersed on a sterile Millipore filter (pore size 0.45 µm) placed on the levelled

soil surface. The culture was wrapped with aluminum foil, placed in a polyethylene bag and incubated at either room temperature or 3°C.

The methods employed to examine parasitism of microsclerotia and endocellular chlamydospores of *A. brassicae* by *N. inventa* were the same as those used in Section II.A, with the following exceptions: Microsclerotia and conidia containing chlamydospores were collected from the periphery of colonies of *A. brassicae* which had been kept at 3°C for 2-3 months. In the preparation of material for scanning electron microscopy, only the freeze-drying technique was used.

RESULTS

Transformation of *Alternaria* conidia to microsclerotia occurred on some naturally infected, partially decayed rapeseed leaves which had been stored at 3°C for 2 to 3 weeks, but not on infected green leaves after storage at 3°C for 3 months. On the partially decayed leaves, there was a profuse growth of contaminating fungi, and a detailed study on the development of microsclerotia was difficult. Therefore, the process was followed only on agar media.

Microsclerotia formed after 2-week-old cultures on V-8 juice agar medium were exposed to 3°C, but not at 25°C. Microsclerotia developed preferentially at the periphery of the colonies.

The stages in the formation of microsclerotia are shown in Plates 12 and 13. Development of a microsclerotium was initiated in one or more cells of a conidium (Plate 12, B and Plate 13, B and C) within 6 to 10 days after storage at 3°C. At about half-maximal development of a microsclerotium, when individual cells were still clearly visible, this initial consisted of more than 50 cells (Plate 12, C). Microsclerotia reached their maximum size in 2 to 3 months (Plate 12, D). Mature microsclerotia were darkly pigmented, many-celled, usually irregularly spheroidal, and about 60-150 µm in diameter. The rough and irregular nature of their surface was demonstrated by scanning electron micrographs (Plate 13, C-E).

More than 98% of the mature (about 2 months) untreated microsclerotia germinated (Plate 12, E and Plate 13, E). Most of the germ tubes developed into hyphae (Plate 12, E), while a few produced conidia directly.

The frozen-thawed microsclerotia also germinated well, but almost exclusively by producing conidiophores and conidia (Plate 12, F). Occasionally more than 25 conidia were produced directly from a single microsclerotium within 24 hours of incubation.

On V-8 juice agar containing rose bengal, during gradual desiccation of *Alternaria* cultures at room temperature, cell division occurred in many conidia after 2 to 3 days. However, as drying continued, the microsclerotial initials ceased to develop.

Microsclerotia were not formed on Millipore filters placed on soil either at room temperature or at 3°C.

Endocellular chlamydospores were observed in many *Alternaria* conidia exposed to 3°C or to gradual desiccation. Chlamydospores occasionally developed in old conidia without these treatments, but they were formed more abundantly in mature conidia and also in some young conidia (Plate 14, A) following one of the two treatments. The chlamydospores were smaller than the cells within which they developed (Plate 14, B). On the surface of the thick, electron-lucid wall of a chlamydospore, remnants of the degenerated cytoplasm of the original cell were usually present (Plate 14, B and C). Occasionally, an organelle consisting of many membrane tubules was seen in the chlamydospore (Plate 14, C).

Endocellular chlamydospores also often developed in cells of conidia placed on natural soil at room temperature. Within a few days, extensive bacterial growth occurred on

the surface of *Alternaria* conidia and cells which had not developed into chlamydospores became empty.

Microsclerotia were actively parasitized by *N. inventa* within 72-96 hours of incubation (Plate 15, A and B). Although detailed cytological changes in the infected cells of microsclerotia could not be traced with the light microscope because of their dark pigmentation, the mode of parasitism appeared to be identical to that of *Alternaria* conidia by the parasite. Tropic growth of the parasite hyphae toward microsclerotia occurred and upon contact appressorium-like bodies were often formed. However, the frequency of the formation of appressorium-like bodies on the microsclerotia was somewhat lower than that on hyphae and conidia of *A. brassicae*. Hyphae of *N. inventa* were closely associated with the septal areas of microsclerotia (Plate 15, C) and penetration occurred commonly at the septa. In the late stage of parasitism, an intense growth of the parasite hyphae occurred on the microsclerotia and a heavy disintegration of the host cell wall was evident (Plate 15, A and B).

Endocellular chlamydospores appeared to be more resistant to the parasite than the cells of hyphae and conidia; however, most of the infected chlamydospores eventually appeared empty within 96 hours of incubation.

Plate 12

Light micrographs of stages in the development of a microsclerotium from a conidium of *Alternaria brassicae*:

A) Conidium of *A. brassicae*. X 400;

B) Initial stage in the formation of a microsclerotium. X 400;

C) Half-developed microsclerotium consisting of more than 50 cells. X 400;

D) Mature microsclerotium. The beak part of the original conidium is still identifiable. X 400;

E) Germination of a mature microsclerotium to form hyphae. X 400;

F) Germination of a frozen-thawed microsclerotium. Note many new conidia produced directly from the microsclerotium. X 650.

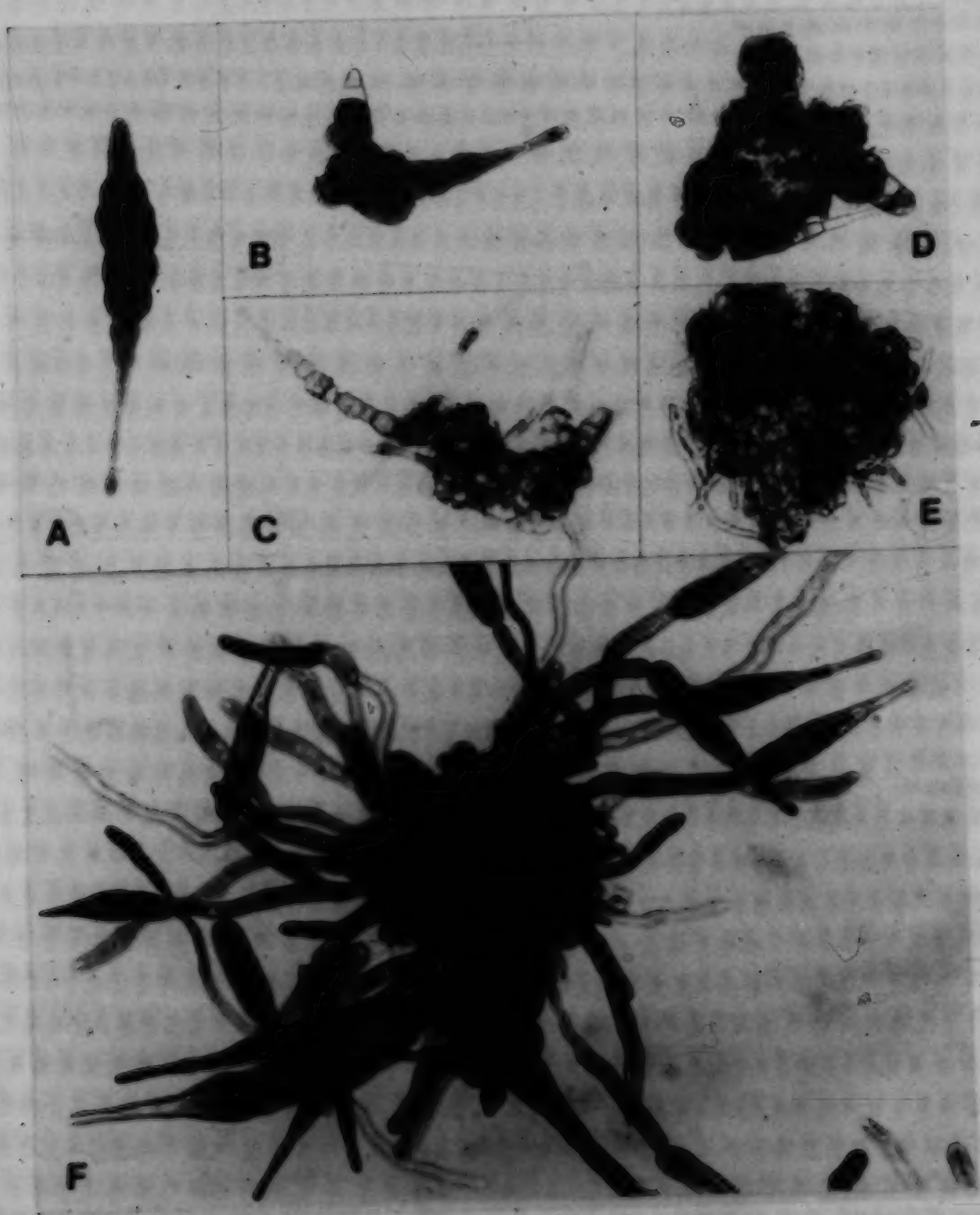

A
B
C
D
E
F

Plate 13

Scanning electron micrographs of stages in the development of a microsclerotium from a conidium of *Alternaria brassicae*:

A) Conidium on a rapeseed leaf. X 1000;

B) Microsclerotium (arrow) developing from a cell of a conidial beak. X 1400;

C) Initial stage in microsclerotium formation. X 2500;

D) Mature, many-celled microsclerotium. X 1400;

E) Magnified view of a part of the microsclerotium in D) showing the emergence of a germ tube. X 12000.

A
B
C
D
E

Plate 14

Formation of endocellular chlamydospores in conidia of *Alternaria brassicae*:

A) Light micrograph of chlamydospores (arrow) formed inside a young conidium. X 750;

B) and C) Transmission electron micrographs of chlamydospores formed inside conidia. Note remnants of degenerated cytoplasm of the original cells and an organelle consisting of many membrane tubules in the chlamydospore in C). B), X 5500, C), X 20000.

Legend: C = remnants of degenerated cytoplasm; M = organelle consisting of many membrane tubules.

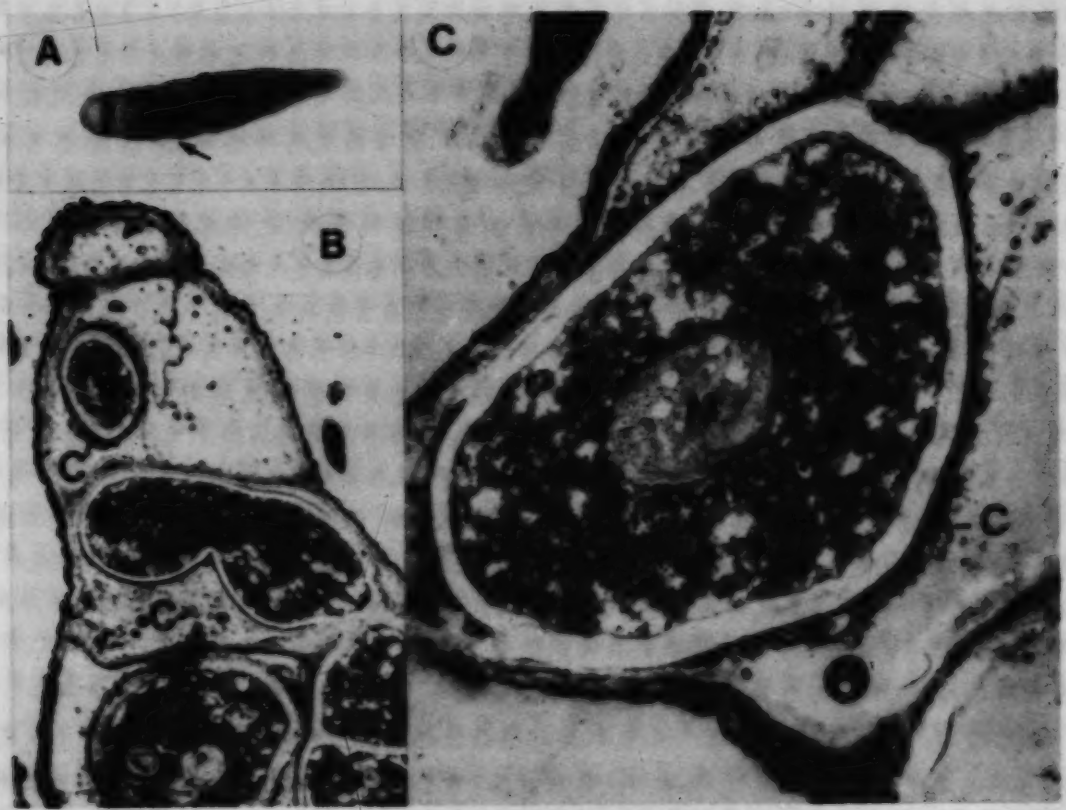
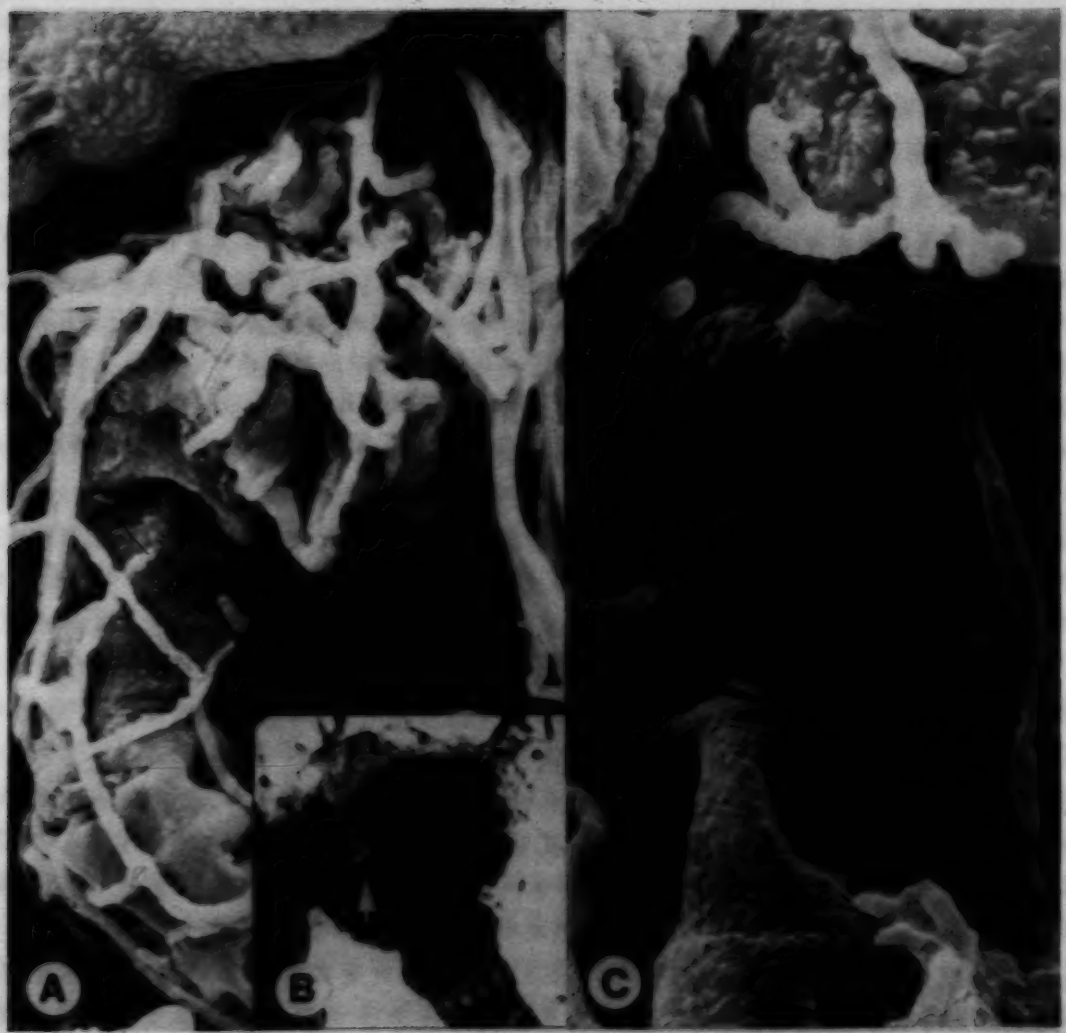

A
B
C
C
C

Plate 15

Microsclerotia of *Alternaria brassicae* attacked by *Nectria inventa*:

A) and B) Scanning electron and light micrographs showing a profuse growth of *N. inventa* on microsclerotia. Degradation of the host cell wall is evident in B) (arrow). A), X 3000, B). X 1300;

C) Close association of *Nectria* hyphae with the septal part of microsclerotium. X 4800.


A
B
C

DISCUSSION

Ainsworth (1971) defined a sclerotium as a firm, frequently rounded mass of hyphae with or without the addition of host tissue or soil, and normally having no spores in or on it. A majority of sclerotia arise as discrete initials among the vegetative hyphae. These initials usually enlarge by hyphal tip growth, and maturation is characterized by surface delimitation and internal consolidation (Butler, 1966). Some of the smallest structures thus formed, consisting of a few cells and without a rind and medulla, are sometimes described as "bulbils" or "microsclerotia". Microsclerotia of *Verticillium albo-atrum* originate from swollen hyphal cells of single or intermingled hyphae and increase in size by budding from globose cells (Brown and Wyllie, 1970). *Pleiochaeta setosa* (Kirchn.) Hughes has also been reported to produce microsclerotia by a similar process (Harvey, 1975). Microsclerotium-like structures in *A. brassicae* are unique in that they are transformed conidia; it may therefore be debatable whether these structures should be called microsclerotia. However, as these structures are firm, rounded, darkly pigmented and resistant to desiccation and freezing, they are functionally similar to sclerotia, and a new term does not seem to be warranted.

Low temperature appears to be necessary for the maximal development of microsclerotia in *A. brassicae*.

However, the fact that microsclerotia were not found on infected green leaves but developed on partially decayed leaves suggests that in nature some other factors may also be involved. Similarly, *Cylindrocladium* spp. do not form microsclerotia on attached azalea leaves but produce them abundantly within two weeks after abscission of the infected leaves (Linderman, 1973).

Initiation of transformation of conidia into microsclerotia also occurred in gradually desiccating *Alternaria* cultures growing on a medium to which rose bengal was added. Rose bengal restricted the vegetative growth of *A. brassicae* but resulted in an increased number of microsclerotia at 3°C. These results suggest that formation of microsclerotia occurs under conditions unfavorable for vegetative growth.

Microsclerotia in *A. brassicae* withstood the effects of desiccation and freezing. This indicates their potential importance in the survival of the fungus, particularly in northern latitudes.

The germination of a frozen-thawed microsclerotium to produce many new conidia (Plate 12, F) suggests the importance of this phenomenon in increasing inoculum density in the spring.

Endocellular chlamydospores, which resembled those reported in *A. porri* f. sp. *solani* (Basu, 1971) were also produced in response to cold temperature and gradual desiccation in culture. Conidia of *A. brassicae* placed on

natural soil formed these thick-walled cells or became empty within one week. This indicates that chlamydospores also have a potential importance in survival of the fungus in nature.

Microsclerotia were destroyed by *N. inventa* in culture. However, in nature their many-celled structure may ensure them a better chance to escape from microbial attacks, including that of *N. inventa*. Environmental conditions fluctuate in nature and, therefore, conditions may not be always favorable for antagonists. Theoretically, survival of only one cell of a microsclerotium is sufficient to re-establish the colony of *A. brassicae* when conditions become favorable for the fungus.

C. FACTORS INFLUENCING THE *ALTERNARIA BRASSICAE* - *NECTRIA INVENTA* HOST-PARASITE INTERACTION

INTRODUCTION

Environmental factors, especially temperature and moisture, undoubtedly play an important role in affecting various microbial activities. Fluctuations in these factors are common especially during the growing season. Combinations of factors such as alternate periods of wetting and drying could also have significant effects on the inocula of certain fungi. In several mycoparasitic host-parasite combinations it was shown that susceptibility of the host fungus varies considerably with temperature (Butler, 1957; Slifkin, 1961; Barnett, 1964). Smith (1972a-c) showed that sclerotia of *Sclerotinia* spp. and *Sclerotium* spp. could be eliminated from soil if they were dried for a short period (1-20 hours) at 30% relative humidity. This treatment caused nutrients to leak from sclerotia and thus promoted their degradation by microbes. Skoropad (1966) reported for *Rhynchosporium secalis* (Oud.) Davis that new batches of conidia were produced from mycelium in lesions only if these lesions were dried and rewetted. During preliminary experiments in this investigation it was found that dried conidia of *Alternaria brassicae* leaked nutrients after they were rewetted.

The purpose of this study was to determine: (1) The effect of different temperatures on the *A. brassicae* - *Nectria inventa* interaction and, (2) The effect of drying and rewetting of conidia of *A. brassicae* on the leakage of nutrients from them and, the significance of these nutrients in the parasitism of *A. brassicae* by *N. inventa*.

MATERIALS AND METHODS

Materials and evaluation methods on the degree of parasitism were the same as used in Sections I.B and II.A. Dual cultures of *A. brassicae* and *N. inventa* were incubated on PDA and on water agar at different temperatures, *viz*, 5, 10, 15, 20, 25 and 28°C. The degree of parasitism was evaluated after incubation for 2 weeks for those grown on PDA, and after 72 hours for those grown on water agar. The effect of temperature on the growth of both fungi in pure culture was examined by measuring their radial growth on PDA after a 12-day incubation period at the temperatures listed above.

Conidia of *A. brassicae* were collected from 8 petri dishes of 2-week-old cultures as described in Section I.B. They were washed in a Büchner funnel, followed by 2 centrifugations at 2000 r.p.m. for 5 minutes each to eliminate any exogenous nutrients carried by conidia from the culture medium. The washed conidia were suspended in 16 ml of sterile distilled water, and 2 ml of the suspension was passed through a sterile Millipore filter (pore size 0.45 µm). Eight Millipore filters retaining conidia were thus obtained. They were treated as 1 sample, which was a basic unit throughout this experiment.

To investigate the effects of dry treatment on *Alternaria* conidia, duplicate samples were dried either in an incubator at 20°C at a relative humidity (RH) of 30 ± 5%, or in deep petri dishes (diameter, 100 mm; height, 80 mm) containing 250 ml of saturated $CaCl_2 - 6H_2O$ (RH, 32%) at 20°C (Clayton, 1942). In the latter case, 2 Millipore filters retaining conidia were placed in a sterile plastic petri dish (60 x 15 mm) which had been placed on the surface of saturated $CaCl_2 - 6H_2O$ solution. The deep petri dish was then sealed with masking tape and kept in a closed polyethylene bag. After drying for 0, 2, 4, and 12 hours, a small sample of conidia was removed and placed on a sterile Millipore filter to test their germinability. The conidia were moistened with sterile distilled water and were incubated in a petri dish moist chamber for 24 hours at 25°C. Eight hundred conidia of each sample were counted.

Each of the dried samples was immersed in 100 ml of sterile distilled demineralized water for 24 hours at 3°C. Conidia were removed and the dry weight of each sample was recorded. The leachate of dried conidia was dried using a rotary evaporator under vacuum at 40°C, and made up to 10 ml in distilled demineralized water. The concentrated leachate was separated into 2 fractions for amino acid and sugar analyses using the method of Sane and Zalik (1968). Each fraction was dried under vacuum at 40°C and made up to a desired volume in distilled demineralized water, unless otherwise noted, and held for analyses.

For quantitative analyses of total free amino acids and reducing sugars, the methods of Rosen (1957) and Nelson (1944) were used, respectively.

Only the leachates obtained from conidia dried for 0 hour (as control) or 12 hours were used for qualitative analysis of amino acids and sugars. The amino acid fraction described above was made up to 5 ml in 0.2 N sodium citrate, pH 2.2, and 1 ml of this solution was analyzed with a Beckman/Spinco model 120 amino acid analyzer (accuracy ± 3%). The amino acids were eluted with citrate buffers of pH 3.25, 4.25 and 5.28 (Miller, 1965). In this procedure the amides, glutamine and asparagine, were eluted together with serine and threonine. The method of Sane and Zalik (1968) was used to obtain resolution of these compounds.

Sugars were determined qualitatively using paper chromatography with n-butanol - acetic acid - water, 4: 1: 5 (v/v/v) as solvent (Partridge, 1948).

To examine the effect of the leaked nutrients on *N. inventa*, conidia of *A. brassicae* were collected and washed as described above. Conidia were suspended in sterile water, and the spore density was adjusted to about 5×10^5 spores/ml. One ml of this suspension was passed through a sterile Millipore filter under vacuum. The Millipore filters bearing conidia were then dried for 6 hours at 32% RH. After drying, the Millipore filters were transferred to sterile microscope slides placed in sterile plastic petri dishes lined with moist filter paper. A drop of sterile distilled water was placed on the edge of each Millipore filter to wet the conidia. A small drop of spore suspension of *N. inventa*, which was made as described in Section I.B, was then placed in the center of each Millipore filter in such a way that the central area consisting of the conidial mixture of both fungi was surrounded by the area containing only *Alternaria* conidia.

A similar experiment was conducted on sterile microscope cover glasses (22 x 22 mm) to confirm the results obtained on Millipore filters. Equal volumes of the above described spore suspensions of each fungus were mixed. About 0.05 ml of the mixture was spread on each cover glass and dried at 32% RH for 0 hour (as control) or 6 hours. These cover glasses were placed in petri dish moist chambers

and the dried conidia were rewetted with about 0.05 ml of sterile distilled water.

For comparison, the manner of germination of non-dried *Alternaria* conidia was examined on Millipore filters wetted with a nutrient rich solution, containing 2% malt extract and 3% sucrose. In all cases, conidia were incubated at 25°C in the dark for 24 hours.

RESULTS

Nectria inventa overgrew and parasitized *A. brassicae* at 20, 25, and 28 °C; however, at 15°C parasitism proceeded slowly on both media used (Table V). *Nectria inventa* did not grow on either culture medium at either 5 or 10°C, while *A. brassicae* grew at a low rate (Table IV). Interaction of these fungi, therefore, did not occur at these temperatures. The optimum temperature for growth of both fungi on PDA appeared to be about 25°C.

Analyses of free amino acids and reducing sugars were done 3 times. The results for the replicates were nearly identical. Therefore, data obtained from one experiment were adopted as a representative of the replicates.

Total amounts of leaked amino acids and reducing sugars from *Alternaria* conidia which were dried for 0, 2, 6, and 12 hours are shown in Table VI. Although small amounts of both amino acids and sugars were detected in the leachate from non-dried conidia (control), their leakage increased markedly when conidia were dried and rewetted; the longer the drying period the larger the amount of leakage.

The exudate from conidia dried for 12 hours consisted of more than 17 different amino acids (Table VII). Among them, glutamine was the largest in amount, followed by aspartic acid and glutamic acid. These were the only amino acids found in the leachate of non-dried conidia (control) and they occurred only in trace amounts. Serine and alanine also formed large proportions of the amino acid fraction, i.e., 10.29 and 9.49% respectively. Other amino acids occurred in relatively low concentrations.

The sugar fraction of the leachate from the dried conidia consisted mainly of glucose (Rf: 0.174) and fructose (Rf: 0.225). In addition, a faint spot (Rf: 0.116) was also present, but it was not identified.

The effect of the leaked nutrients on *Nectria* conidia was striking; 61.7% of them germinated in the vicinity of the dried and rewetted *Alternaria* conidia within 24 hours on Millipore filters (Plate 16, A and C), while only 1-2% of *Nectria* conidia germinated around non-dried *Alternaria* conidia (Plate 17, B). The stimulating effect of the leaked nutrients on the germination of *Nectria* conidia was also evident in the experiment using microscope cover glasses (Plate 17, A).

Germinability of *Alternaria* conidia was reduced only slightly by dry treatment; germination rates of the conidia dried for 0, 2, 6 and 12 hours were 99.2, 98.0, 97.1, and 93.7% respectively. However, a few cells, especially the

proximal and its adjacent cells, of the dried conidia often appeared empty when rewetted. The number of the empty-appearing cells increased only moderately with longer drying periods.

The dry treatment brought about marked changes in the response of *Alternaria* conidia to *Nectria* on Millipore filters wetted with sterile distilled water. When surrounded by *Nectria* conidia, less than 5% of the dried *Alternaria* conidia germinated after 24 hours of incubation (Plate 16, A and C). They were eventually destroyed by *N. inventa*, while the majority of them (>95%) germinated where *Nectria* conidia were absent (Plate 16, A and B). Non-dried conidia of *A. brassicae*, however, readily germinated in the presence of *Nectria* conidia.

The manner of germination of *Alternaria* conidia also changed after the dry treatment. On Millipore filters wetted with sterile distilled water, only about 32% of non-dried *Alternaria* conidia germinated by producing germ tubes which developed into hyphae, while 65.8% of them germinated by producing secondary conidia directly (microcyclic conidiation) (Plate 17, B). A few of them produced both secondary conidia and vegetative germ tubes. In contrast, a majority of the dried conidia germinated by means of vegetative germ tubes (Plate 16, B), and the rate of microcyclic conidiation dropped considerably to 20.3%.

Germination of non-dried *Alternaria* conidia was examined on Millipore filters wetted with a solution

containing 2% malt extract and 3% sucrose. In the presence of the exogenous nutrients, *Alternaria* conidia germinated exclusively by producing vegetative hyphae (Plate 17, C).

TABLE IV. The effect of different temperatures on the growth of *Alternaria brassicae* and *Nectria inventa* on potato-dextrose agar medium.

Temperature (C)	Radial growth after 12 days (mm)[1)]	
	A. brassicae	*N. inventa*
5	4.1	0
10	4.6	0
15	11.6	23.5
20	19.9	36.9
25	31.9	45.5
28	30.7	43.3

[1)] Average of 4 replications for each fungus.

TABLE V. Degree of parasitism of *Alternaria brassicae* by *Nectria inventa* at different temperatures.

Temperature (C)	Colony interactions on PDA (Scores for *A. brassicae*)[1)]	Index of host susceptibility on water agar[2)]
5	-[3)]	-[3)]
10	-	-
15	2	S
20	2	VS
25	2	VS
28	2	VS

[1)] See Fig. 1.

[2)] S = moderately susceptible, VS = very susceptible.

[3)] Growth of *N. inventa* did not occur.

TABLE VI. The amount of leakage of sugars and amino acids from conidia of *Alternaria brassicae* dried for different periods of time.

Period of drying (hr)	Sugars[1] (μ moles/100 mg dry weight conidia)	Amino acids[2] (μ moles/100 mg dry weight conidia)
0	0.68	0.25
2	5.60	3.54
6	16.48	8.84
12	23.60	12.05

[1] Expressed as glucose.

[2] Expressed as leucine.

TABLE VII. Amino acids leaked from conidia of *Alternaria brassicae* dried for 0 or 12 hours.

Amino acid	µ moles/100 mg dry weight of conidia period of drying (hr) 0	12	(% concentration of each amino acid)
Lysine	-[1]	0.619	(5.70)
Histidine	-	0.234	(2.15)
Arginine	-	0.391	(3.60)
Aspartic acid	trace	1.548	(14.25)
Threonine	-	0.582	(5.36)
Serine	-	1.118	(10.29)
Glutamic acid	trace	1.516	(13.95)
Proline	-	trace	(/)
Glycine	-	0.420	(3.87)
Alanine	-	1.031	(9.49)
Half Cystine	-	trace	(/)
Valine	-	0.220	(2.02)
Methionine	-	0.086	(0.79)
Isoleucine	-	0.191	(1.76)
Leucine	-	0.422	(3.88)
Tyrosine	-	0.210	(1.93)
Phenylalanine	-	0.185	(1.70)
Asparagine	-	0.245	(2.25)
Glutamine	trace	1.847	(17.00)

[1] Nil or non-measurable.

Plate 16 and 17

Effects of dry treatment of conidia of *Alternaria brassicae* on their germination and on the interaction with *Nectria inventa*:

Plate 16

A) Profuse growth of *N. inventa* in the vicinity of *Alternaria* conidia which were dried for 6 hours and rewetted on a Millipore filter. The broken line indicates an approximate border between an area containing only *Alternaria* conidia (left side) and an area where *Nectria* conidia were added (right side). Most of the *Alternaria* conidia (right side) do not germinate where *Nectria* is growing. X 150;

B) Germination of the dried conidia by means of vegetative hyphae. Cytoplasm of these conidia appears to become intracellular hyphae during germination. X 600;

C) Magnified view of *Nectria* conidia growing in the vicinity of *Alternaria* conidia which were dried for 6 hours and rewetted. X 300.

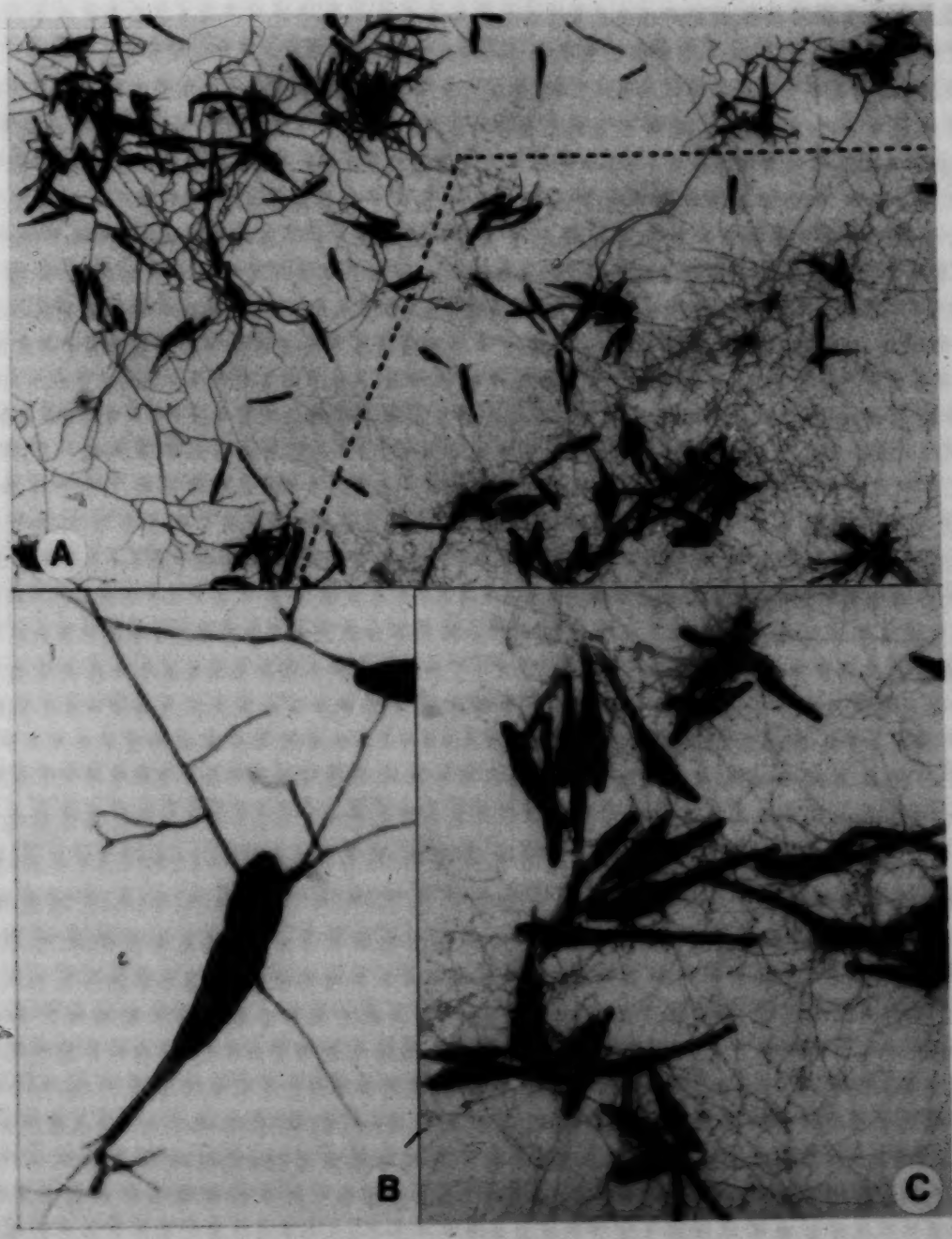
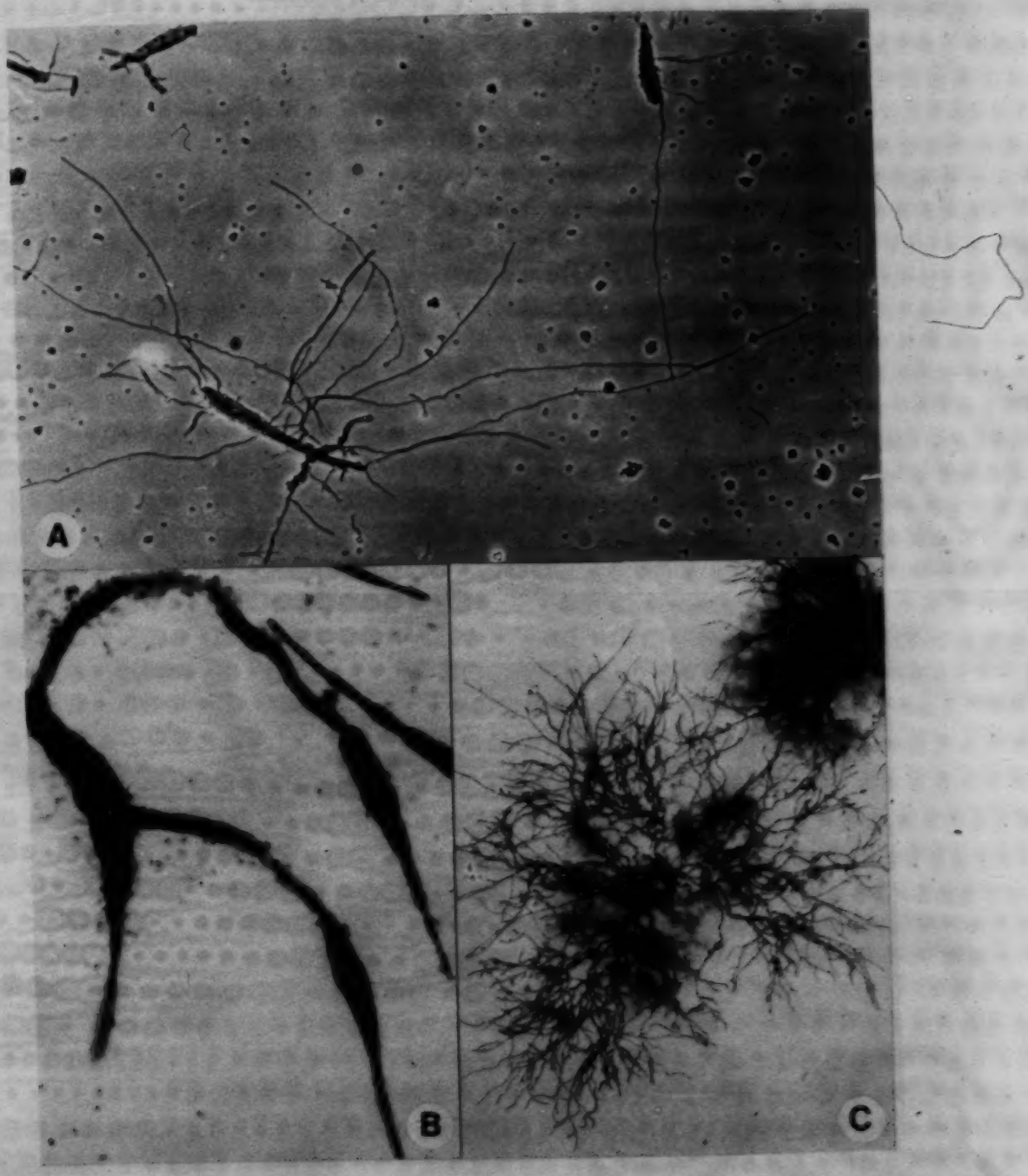

A
B
C

Plate 17

A) Active germination of *Nectria* conidia on and in the vicinity of *Alternaria* conidia which were dried for 6 hours and rewetted on a microscope cover glass. *Nectria* conidia situated at a distance from the *Alternaria* conidia seldom germinate. X 300;

B) Microcyclic conidiation by a non-dried *Alternaria* conidium on a Millipore filter wetted with distilled water. Note poor germination of *Nectria* conidia. X 600;

C) Network of vegetative hyphae produced by a non-dried *Alternaria* conidium on a Millipore filter wetted with a liquid containing 2% malt extract and 3% sucrose. Microcyclic conidiation is absent. X 100.


A
B
C

DISCUSSION

In several mycoparasitic relations, temperature has been shown to be one of the most important factors which determine the degree of parasitism (Butler, 1957; Slifkin, 1961; Barnett, 1964). This also applies to the *A. brassicae* - *N. inventa* interaction. Parasitism was strong at 20, 25, and 28°C, moderate at 15°C and absent at 10 and 5°C. The ability of *A. brassicae* to grow at low temperatures is advantageous to the fungus to escape from mycoparasites and to increase inoculum density in early spring.

Spores of several fungi have been reported to exude nutrients when suspended in distilled water (Lingappa and Lockwood, 1964) or when placed on plant leaves (Fraser, 1971) or on soil (Bristow and Lockwood, 1975). Although non-dried conidia of *A. brassicae* leaked minute amounts of nutrients in distilled water, the amount of leakage increased markedly after dry treatment. Dry treatment has also been shown to cause leakage of exudates from sclerotia of several fungi (Smith, 1972a-c), and from roots of higher plants (Katznelson, Rouatt, and Payne, 1955) when the soil is subsequently rewetted. The mechanisms which induce these phenomena are not well known; in the former case, however, it was attributed to cracks which developed in the rind of

sclerotia after drying. A similar explanation may apply to conidia of *A. brassicae*. As described in Section II.A, the conidial cell wall of *A. brassicae* consists of 4 layers. The outermost layer of a conidium (C_1 in Plate 8, C) appears to be lipid in nature because it is hydrophobic and electron dense. Therefore, the function of this layer is expected to be similar to that of epicuticular wax of higher plants. Martin (1964) suggested that wax impedes either the movement of leaf exudates or the passage of water from the leaf surface through the cuticle. It is likely that the continuity of the outermost layer of *Alternaria* conidia is broken by shrinkage of the cell wall during drying, followed by a sudden expansion when rewetted. This could result in the removal of the important barrier and a subsequent increase in leakage.

Excessive drying may also damage the plasma membrane and cellular contents of *Alternaria* conidia. Cook and Papendick (1972) suggested that the harmful effects of desiccation on propagules of plant pathogens are partly attributed to the effects on bound water in macromolecules of the cell, including proteins, enzymes, RNA, and DNA. This is also likely to occur in *Alternaria* cells during drying and consequently causes weakening and death of the cells. Since control of cell permeability by the plasma membrane is an energy-requiring process, the declined viability of the *Alternaria* cells may be accompanied by an

increased leakage of endocellular compounds through the plasma membrane. Leakage occurs freely from dead cells of *Alternaria* conidia. The empty-appearing cells, which often occurred in the dried and rewetted *Alternaria* conidia, are probably dead cells, and thus they are an important source of the leaked nutrients. However, since the number of empty-appearing cells increased only moderately with longer drying periods, this phenomenon alone cannot explain the drastic difference in the amount of the leaked nutrients between 2-hour-dried and 12-hour-dried samples (Table VI). Therefore, the leakage of nutrients probably resulted from a combination of the above described mechanisms.

A pore is present at the base of the proximal cells where the thick wall is lacking. The fact that these cells were most susceptible to the dry treatment suggests that the thick cell wall plays an important role in protecting conidial cells from desiccation.

The composition of amino acids and sugars which leaked from dried *Alternaria* conidia is similar to those of the ethanol extracts of hyphae of *A. triticina* Prasada & Prabhu and *A. tenuis* (= *A. alternata*). These extracts contained glucose, fructose and more than 10 free amino acids (Kumar and Rao, 1976).

Phipps and Barnett (1975) suggested that the concentration of specific amino acids in the free amino acid pool of the host is a major factor in determining the degree

of parasitism by biotrophic haustorial mycoparasites. However, this may not be applicable to the *A. brassicae* - *N. inventa* interaction. Considering that *N. inventa* has a relatively wide host range (unpublished data) and also has a strong saprophytic ability on a wide variety of substrata (Hughes, 1951), the stimulating effect of the leaked nutrients on the germination of *Nectria* conidia is unlikely to be specific.

Dried and rewetted *Alternaria* conidia retained a high germinability but most of them failed to germinate in the presence of *Nectria* conidia nearby. A similar phenomenon has been reported in connection with soil fungistasis. Bristow and Lockwood (1975) demonstrated that nutrient-independent spores of several fungi exude non-specific energy-rich nutrients during incubation on sand being leached with phosphate buffer. As a result they became dependent upon exogenous nutrients for germination. These authors suggested that in natural soil the exuded nutrients may be rendered unavailable to the spores because of microbial activity, thus resulting in fungistasis. This theory may explain why the dried conidia of *A. brassicae* could not germinate near *N. inventa*. A steep diffusion gradient may be created between *Alternaria* and *Nectria* because of the vigorous depletion of the leaked nutrients by the latter. *Nectria*, therefore, induces more leakage from the *Alternaria* conidia and eventually exhausts their nutrients to the extent that they fail to germinate. In the absence of

N. inventa, *Alternaria* conidia may not lose their nutrients too extensively and/or may re-utilize the leaked nutrients sufficiently to be able to germinate.

It is also possible that *N. inventa* produces a weak fungistatic substance which suppresses the germination of *Alternaria* conidia which have been weakened by dry treatment. Although microscopic observation of the *A. brassicae* - *N. inventa* interaction (Section II.A) indicates that *N. inventa* does not produce a diffusible toxic substance that affects normal (non-dried) host cells at any appreciable distance, this does not exclude the above possibility.

Microcyclic conidiation in *A. porri* f. sp. *solani* has been shown to be induced by various treatments which interfere with normal germination of mother spores (Rotem and Bashi, 1969). Skidmore (1976a) suggested that the formation of secondary conidia is related to the nutrient status of the substrate on which the mother spore germinates. Microcyclic conidiation observed in the present study appears to be a response to the lack of exogenous nutrient, since it did not occur in the presence of rich exogenous nutrient.

D. BEHAVIOR OF *ALTERNARIA BRASSICAE* AND *NECTRIA INVENTA* ON INTACT AND ON EXCISED RAPESEED LEAVES

INTRODUCTION

A serious problem in the biological controls has been in the discrepancy between the results obtained *in vitro* and those obtained *in vivo*. For example, Bhatt and Vaughan (1963) showed that *Penicillium* sp. was the most effective fungus to suppress growth of *Botrytis cinerea* on agar, but it had no effect on the same fungus *in vivo*. Likewise, *Penicillium* sp. was strongly antagonistic to *Alternaria zinniae* on agar, but had no effect on this fungus on leaves (Heuvel, 1970).

Nectria inventa, as a biological control agent, also presents some special problems. Preliminary field experiments showed that *N. inventa* does not suppress primary infection of intact leaves by *A. brassicae*. In these experiments, leaves of Midas and Torch, grown in the field, were spray-inoculated with spore suspensions containing either *A. brassicae* alone or a mixture of *A. brassicae* and *N. inventa*. There were no visible differences in the extent of infection caused by the two kinds of inoculum.

The present study was conducted to determine why *N. inventa* failed to suppress *A. brassicae* on intact rapeseed leaves, and also to examine the effectiveness of *N. inventa* against *A. brassicae* on detached leaves.

MATERIALS AND METHODS

Cultivars of rapeseed, Midas and Torch, were grown in soil in 18 cm pots in a growth chamber at 21-22°C and 14 hours light (12000-15000 lux). The third and fourth true leaves of the plants were used when they were 35-40 days old in Torch and 45-50 days old in Midas. Half a leaf to one side of the midrib was spray-inoculated with a conidial suspension of *A. brassicae* and the other half with a suspension containing conidia of *A. brassicae* and *N. inventa*. The preparation of these spore suspensions was the same as that used in Section II.A. Approximately 0.3 ml of either suspension was sprayed on each half leaf. The plants were covered with a polyethylene bag and were kept in the growth chamber under the same conditions as described above. Four inoculated leaves were harvested periodically, and the behavior of both fungi on the leaves was examined under light and scanning electron microscopes.

For observation with a light microscope, Sellotape impressions (Edwards and Hartman,1952) and leaf clearing methods were used. In the first method, conidia on the harvested leaves were removed with Sellotape by impressing its adhesive side to the leaf surface. This method was used primarily to examine the germination stage of conidia. In the second method, leaves were cut into pieces (about

15 x 15 mm) and then immersed in a decolorizing solution consisting of equal parts (volume/volume) of glacial acetic acid and absolute ethanol until the tissues had cleared. The Sellotape strips and the leaf pieces carrying conidia were mounted in cotton blue-lactophenol on microscope slides for examination. In each method, 800-1000 conidia of each fungus were examined and the same experiment was done 3 times.

For scanning electron microscopy, 7 mm discs were cut at random from the harvested leaves and then fixed and freeze-dried using the method described in Section II.A.

To examine the interaction between *A. brassicae* and *N. inventa* on excised rapeseed leaves, the third and fourth true leaves were harvested (4 leaves for each cultivar) from plants grown as described above. Half-leaf inoculation was done using the same kind of spore suspensions as used above. One side of each leaf was inoculated at two separate spots with *A. brassicae* alone by placing a small amount (about 0.05 ml) of spore suspension, and the other side was inoculated with a mixed suspension of both fungi. Half a batch of these leaves was placed in petri dish moist chambers immediately after inoculation, while the other half of the batch was used to investigate the effect of dry treatment on the interaction of the 2 fungi. The inoculated leaves were left at room conditions (22-25°C; 20-40% RH) for 15-20 hours. A drop of sterile distilled water was then placed on each spot where

inoculum had been placed, and the leaves were transferred into moist chambers. The leaves were incubated in the growth chamber, described above, and the growth of both fungi on the leaves was examined periodically with a stereomicroscope over a period of 2 weeks. This experiment was done 3 times.

RESULTS

On intact leaves, conidia of *A. brassicae* germinated after a short incubation period (Plate 18, A). The germination rates on Midas and Torch were 12.1% and 19.5% at 3 hours after inoculation, and 36.7% and 45.5% at 9 hours, respectively. They germinated usually by producing either germ tubes or secondary conidia, but a few produced both structures (Plate 18, B). The Sellotape impression technique showed that at 9 hours 21.3% and 16.5% of all the germinated conidia did so by means of microcyclic conidiation on Midas and Torch, respectively. At this stage, conidia of *N. inventa* did not germinate (Plate 18, A).

Simple (single-celled) appressoria occurred in the terminal and intercalary positions (Plate 18, C, and Plate 19, C). Appressoria often occurred at random locations on the leaf surface of both cultivars, but occasionally they were closely associated with cell junctions and stomata (Plate 18, C).

Penetration of leaves by *A. brassicae* was evident at 12 hours, and was abundant at 24 hours. It occurred either with or without appressoria (Plate 18, D, and Plate 19, A, C, and D). The manner of penetration was different on each rapeseed cultivar. Hyphae of *A. brassicae* penetrated Torch leaf tissue either directly through epidermal cells (Plate 18, D) or indirectly through stomata (Plate 19, A and D) at a rate of 21.8% and 78.2%, respectively at 48 hours. Penetration of Midas leaves by *A. brassicae* was almost exclusively through stomata. The frequency of direct penetration was extremely low, being less than 1% of all the penetrations examined. Penetration of stomata occurred by entry of either the main germ tube or a lateral branch, which often appeared to grow tropically toward the stoma (Plate 19, A and D). However, this positive tropism did not occur consistently; sometimes side branches grew away from the stoma (Plant 19, E). Necrosis of the host leaf tissue was apparent around some *Alternaria* conidia at 24 hours.

A few *Nectria* conidia (less than 2%) began to germinate at 24 hours after inoculation (Plate 19, A and B), but growth of the germ tubes was slow and consequently parasitism of *A. brassicae* by *N. inventa* was not seen.

At 48 hours after inoculation, most *Alternaria* conidia germinated. The germination rates on Midas and Torch leaves were 80.5% and 87.4%, respectively and of all the germinated conidia, 28.1% and 18.1% did so by means of micro-cyclic conidiation, respectively. At this stage, a few secondary conidia initiated germination and a number of the internal hyphae reappeared mostly through stomata (Plate 20, A). In some instances the emerging hyphae formed conidia (Plate 20, B).

At 48 hours after inoculation, the germination rates of *Nectria* conidia were 6.3% and 3.5% on Torch and Midas, respectively. At 4 days, their germination rates increased to 28.5% and 16.8% on the respective cultivars, and occasionally parasitism of *A. brassicae* by *N. inventa* was observed. However, by this time, the blackspot lesions of the leaves were fully developed and newly formed *Alternaria* conidia were abundant.

On excised leaves *N. inventa* behaved differently. On the half leaves on which the mixed spore suspension was placed, *N. inventa* began to sporulate within 48 hours after inoculation, and formed a thick brick-red conidial mass within 4-6 days. Subsequently, vegetative growth and sporulation of *A. brassicae* were drastically inhibited.

In the absence of *N. inventa*, *A. brassicae* grew profusely and established a white mycelial mat within 4-6 days. After 2 weeks of incubation, the half leaves which had been inoculated with both fungi were entirely covered by *N. inventa* and the fungus began to invade the other half of the leaves on which *A. brassicae* had sporulated abundantly.

Dry treatment of inoculated excised leaves enhanced growth and sporulation of *N. inventa* in areas where a mixed spore suspension was placed. Otherwise, the sequence of events was the same as described for non-dried excised leaves.

Plate 18-20

Behavior of *Alternaria brassicae* and *Nectria inventa* on intact rapeseed leaves:

Plate 18

A) Germinating *Alternaria* conidium and ungerminated *Nectria* conidia (arrows) on the leaf surface of Torch at 9 hours after inoculation. X 1500;

B) Microcyclic conidiation by *Alternaria* conidia on Midas at 24 hours. Note the presence of a conidium producing both a vegetative germ tube and a secondary conidium. X 350;

C) Formation of appressoria (arrows) by *A. brassicae* over cell junctions and a stomatal guard cell of Torch at 24 hours. X 500;

D) Direct penetration (arrow) of an epidermal cell of Torch by *A. brassicae* at 24 hours. X 1000.

Legend: GT = vegetative germ tube; PO = pollens of rapeseed; SC = secondary conidium; ST = stoma.

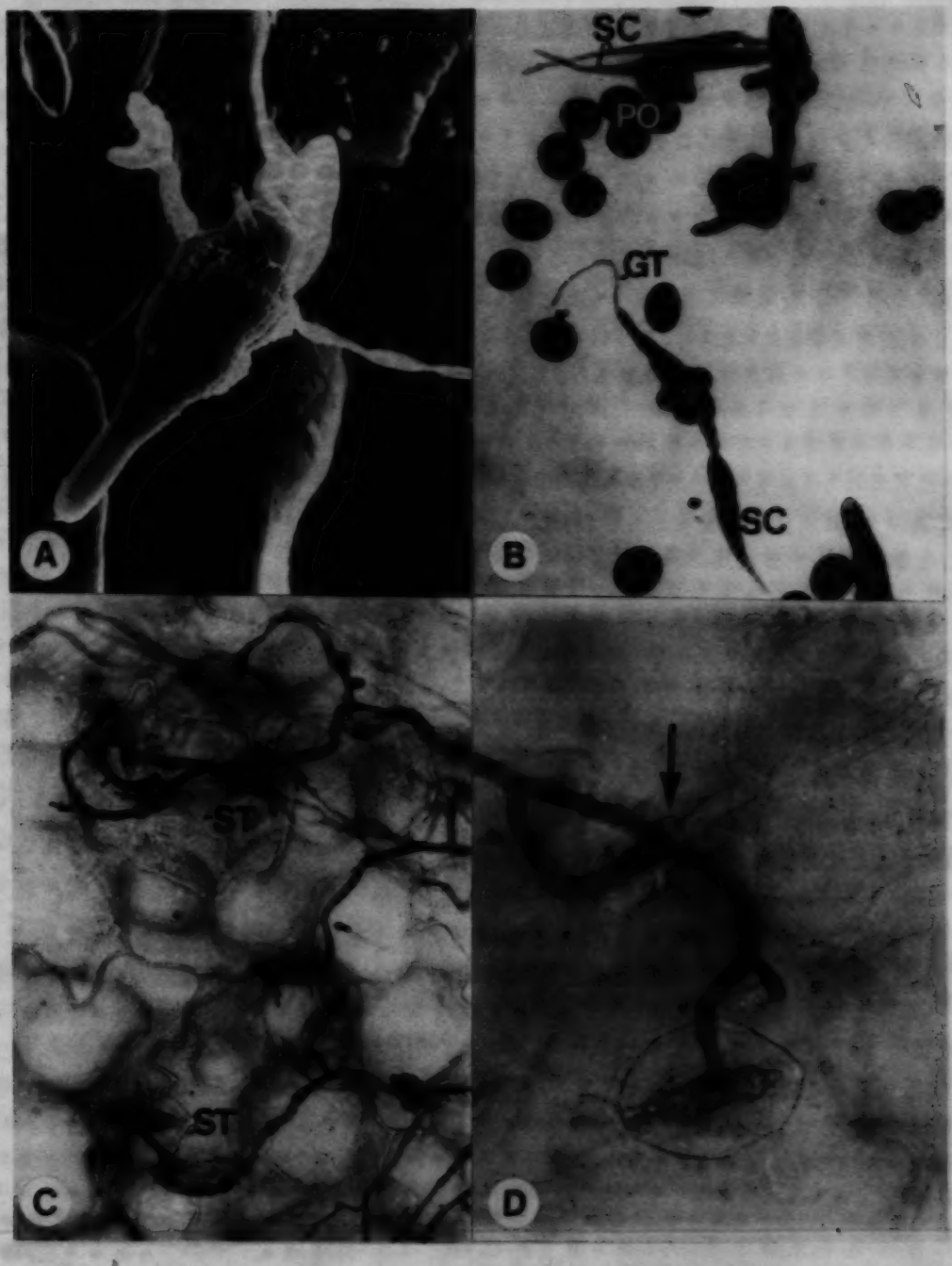

SC
PO
GT
SC
A
B
ST
ST
C
D

Plate 19

A) Penetration through a stoma (arrow) of Torch by *Alternaria brassicae* at 24 hours after inoculation. A few of *Nectria* conidia are germinating. X 1500;

B) Conidia of *N. inventa* on Torch at 24 hours. Two of them are germinating. X 2200;

C) Stomatal penetration from an appressorium (arrow) by *A. brassicae*. The presence of penetration was confirmed by viewing from different angles. On Torch at 24 hours. X 2000;

D) Stomatal penetration (arrow) without forming an appressorium. On Torch at 24 hours. X 6000;

E) Side branch of *Alternaria* hypha growing away from a stoma of Midas at 24 hours. X 1400.

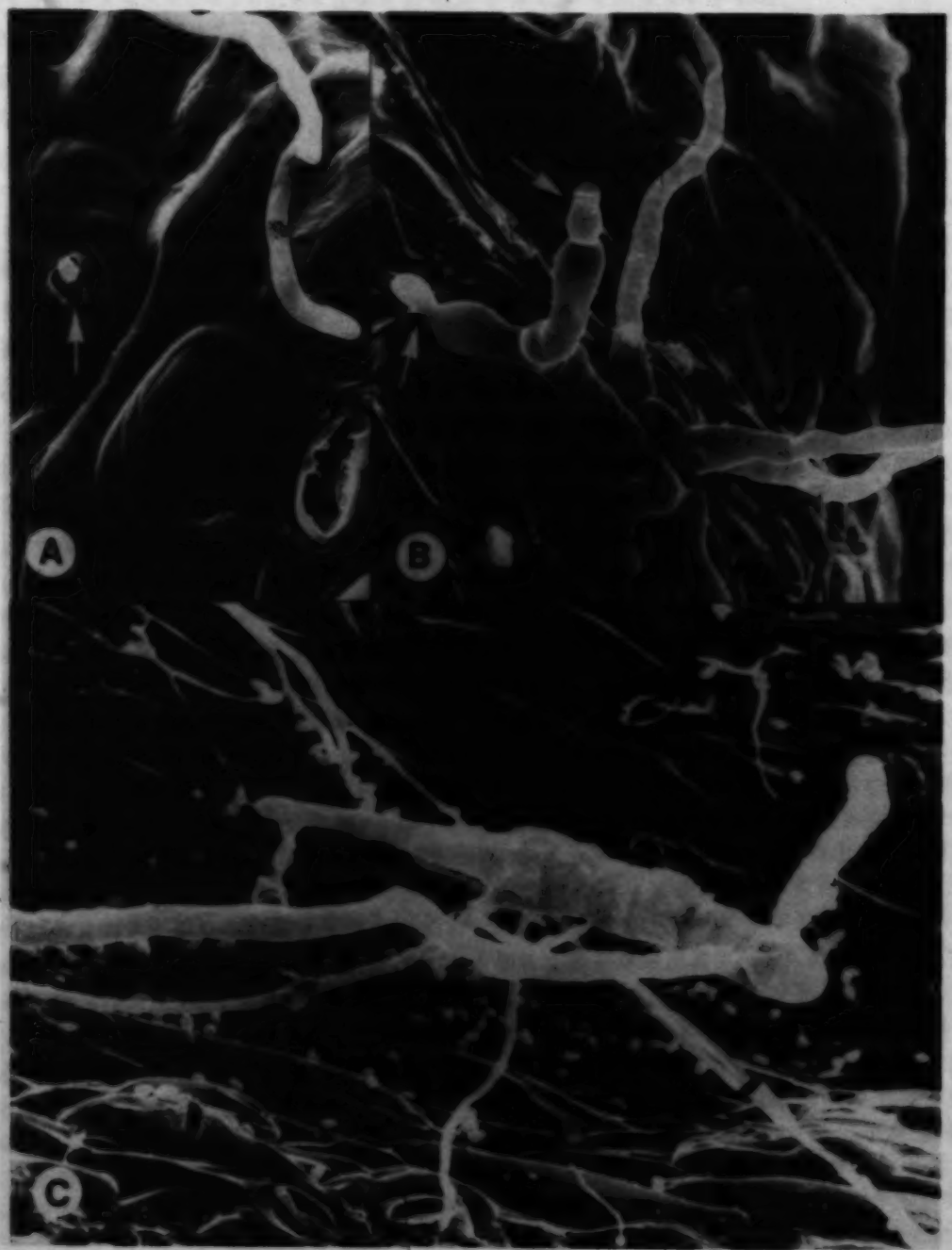
A
B
C
D
E

Plate 20

A) Tip of an internal hypha of *Alternaria brassicae* (arrow) emerging from a stoma of Torch at 48 hours after inoculation. X 1500;

B) Formation of conidia (arrows) over a stoma of Torch at 48 hours. X 2200;

C) Conidia of *Nectria inventa* and *A. brassicae* after a 48-hour incubation on Torch. Germination rate of *Nectria* conidia is still low and germ tubes are short. X 1500.


A
B
C

DISCUSSION

The Sellotape impression technique was the simplest and the most accurate method used to estimate the germination rate of *Alternaria* conidia on leaves, and was especially useful to detect the occurrence of microcyclic conidiation. In using other methods, i.e., leaf clearing and scanning electron microscopy, secondary conidia tended to be detached from the mother conidia and many of them, particularly the non-germinated ones, were removed from the leaf surface during decoloration or fixation procedures.

Alternaria brassicae increases its inoculum density by forming secondary conidia. This phenomenon may also be important in the survival of the fungus in nature, particularly when germinating conidia encounter a sudden change in environmental conditions. A similar conclusion was reached by Skidmore (1976a) who studied microcyclic conidiation in several common phylloplane fungi.

Alternaria brassicae often formed appressoria at random locations on the surface of rapeseed leaves; however, cell junctions and stomata were favored sites for their formation. Some other plant pathogenic fungi also have a strong preference for these sites (Emmett and Parbery, 1975), for example, *Peronospora parasitica* (Preece, Barnes, and Bayley, 1967) and *Uromyces phaseoli* (Pers.) Wint. (Wynn, 1976) form appressoria almost exclusively over cell junctions and stomata. Wynn (1976) suggested that the bean rust fungus

forms appressoria in response to the uneven surface morphology of the host leaves. This may also explain the mode of appressorium formation by *A. brassicae*, since the leaf surface of rapeseed appears to be highly uneven (Plate 19, E).

McDonald (1959) and Changsri and Weber (1963) reported that penetration of leaves of *B. napus* by *A. brassicae* occurs only indirectly through stomata. In the present study, penetration of Midas (*B. napus*) leaves by the fungus was almost exclusively stomatal, and thus it confirms the above authors' results. However, penetration of Torch (*B. campestris*) leaves was often through epidermal cells as well as through stomata. Therefore, the mode of penetration by this fungus differs with host species. This may explain, at least in part, contradictions existing among reports of the mode of penetration by different species of *Alternaria* (Saad and Hagedorn, 1969).

After 12 hours of incubation penetration of rapeseed leaves by *A. brassicae* was evident, and within 48 hours blackspot lesions developed. Conidia of *N. inventa*, however, required a much longer time for germination, and parasitism against *A. brassicae* was observed only after 4 days. Thus, *A. brassicae* escaped *N. inventa* because of its more rapid development.

These results may explain why *N. inventa* did not control the *Alternaria* infection of rapeseed leaves in the preliminary field experiments. But, why does *N. inventa*

require such a long time to initiate parasitism on intact rapeseed leaves? On water agar, the fungus began to parasitize *A. brassicae* within 24 hours (Section II.A). Among several possibilities, the extent to which nutrients are available may be the most important factor involved in this phenomenon. Conidia of *N. inventa* require an exogenous source of energy to germinate, and good vegetative growth of this fungus appears to be a prerequisite for parasitism of *A. brassicae.* The surface of intact rapeseed leaves may lack sufficient nutrients for germination.

Foliar application of *Nectria* conidia is thus an impractical method to control the primary infection of rapeseed by *A. brassicae.* Infection occurs before *N. inventa* becomes parasitically active and when hyphae of *A. brassicae* enter the leaf tissue, the fungus is protected from an attack by *N. inventa* which cannot grow inside living plant tissue.

Nectria inventa, however, suppressed vegetative growth and sporulation of *A. brassicae* on detached leaves. When leaves are excised, the leaf cells undergo a rapid process of senescence. According to Tukey (1971), water droplets present on plant leaves contain various inorganic and organic nutrients originating from the leaf cells, and the amount of nutrients increases with the age of the leaf, reaching a peak at senescence. Therefore, the excised rapeseed leaves probably supply sufficient energy to *Nectria* conidia for their germination.

In the field, *A. brassicae* causes defoliation of infected leaves and the fungus is likely to increase its inoculum density on them. *Alternaria brassicae* is a strong saprophyte and under humid conditions it sporulates abundantly on dead leaves. The *A. brassicae* - *N. inventa* interaction which occurred on the excised leaves indicates that *N. inventa* may be able to prevent the blackspot pathogen from increasing its inoculum density on fallen leaves and other plant debris. On these substrata, there is maximal time for contact and destruction of the pathogen and, therefore, rapidity of germination of *Nectria* conidia is not of prime importance.

On excised leaves, *N. inventa* sporulated abundantly on the spots where a mixed spore suspension was placed. Sporulation was enhanced by dry treatment of excised leaves when the inoculum was subsequently rewetted. This phenomenon probably occurs because of the leakage of endogenous nutrients from the *Alternaria* conidia.

The results of this study suggest that an increase in the population of *N. inventa* induced by artificial means may reduce the inoculum density of *A. brassicae* on rapeseed debris in nature, particularly after a dry period.

CONCLUDING REMARKS

The mycoparasitic interaction between *Alternaria brassicae* and *Nectria inventa* causes a series of cytological changes in the fungal host cells, which are surprisingly similar to those induced in higher plant cells in response to certain fungal parasites. This indicates that, at the cellular level, the nature of parasitism in the plant world may have many aspects in common and that the results are often transferable among different parasitic models.

The mycoparasitic host-parasite system provides a better model than the higher plant - fungal system for studying parasitism because: (1) It requires less time and space for its culture (Manocha and Lee, 1971); (2) The cultural conditions can be easily controlled without using costly devices; (3) Uniformity of materials is superior; (4) Interference from contaminating micro-organisms can be more easily and completely controlled; and (5) Periodic microscopic observation of the progress of parasitism occurring within the same spot is possible, and sectioning of material is not always required to follow cytological changes in the infected host cells.

Observations of the ultrastructural aspects of parasitism were initiated in 1969 (Armentrout and Wilson, 1969; Hashioka and Fukita, 1969). Since 1976 there has been rapid progress in this field (Carling, Brown, and Millikan, 1976; Ikediugwu, 1976a,b; Jeffries and Young, 1976; Tsuneda,

Skoropad, and Tewari, 1976; Hoch and Fuller, 1977; Tsuneda and Skoropad, 1977), and this has had an important impact on the science of plant pathology. For example, Hoch (1977b) discovered a unique means of parasitism; he showed actual cytoplasmic continuity, via plasmodesmata, between *Gonatobotrys simplex* (biotrophic contact parasite) and its host, *A. tenuis* (= *A. alternata*) and suggested that nutrient and growth factors are taken up by the parasite through the plasmodesmata. Investigations of the ultrastructure of an increasing variety of mycoparasitic host-parasite interactions should provide us with a better understanding of the basic principles underlying such phenomena as disease resistance and susceptibility.

The study of the biological control of *A. brassicae* by using *N. inventa* is still in a preliminary stage. Synchronization of certain critical events appears to be the key to a successful biological control of this pathogen. The mycoparasite, in this case, has different nutritional requirements for germination than the host fungus. A knowledge of these requirements, and their artificial adjustment (e.g., the addition of sugars and amino acids at appropriate times) may provide a simple solution to what now appears to be a complex problem. These adjustments may vary according to each substratum, as is illustrated by the different behavior of *Nectria* spores on intact leaves and on detached leaves.

Any artificial modification of environments, however, must be approached cautiously because it may alter other biological relationships and result in a deleterious rather than beneficial overall effect. This is particularly true when the vast and complex sphere of micro-organisms is involved.

At the moment it appears that *N. inventa* can be, and probably is effective in reducing the inoculum load of *A. brassicae* on plant debris. The reduction of primary inoculum is one of the basic measures in controlling a variety of plant diseases.

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